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PRINCIPAL INVESTIGATOR: Lisa A. Palmer, Ph.D.

CONTRACTING ORGANIZATION: University of Virginia
                          Charlottesville, VA 22904

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Lisa A. Palmer, Ph.D.

E-Mail: lap5w@virginia.edu

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13. SUPPLEMENTARY NOTES

14. ABSTRACT
Nitric oxide (NO) transfer reactions between protein and peptide cystines are thought to represent a regulated signaling process. In the current studies, N-acetyl cysteine (NAC) was used as a bait reactant to measure NO transfer reactions in the blood and the vascular effects of these reactions in the pulmonary vasculature. In blood, NAC was converted to S-nitroso-N acetyl cysteine (SNOAC) both in vivo and ex vivo. Ex vivo, SNOAC formation was found to be dependent on oxyhemoglobin desaturation. The formation of SNOAC was found to be a relatively slow ($K \sim 5.3 \times 10\text{-}10 M/s$) pseudo-first order reaction. Physiologically, chronic administration of NAC resulted in the development of pulmonary hypertension that was indistinguishable from that caused by chronic hypoxia. Male endothelial nitric oxide synthase (eNOS) mice were protected from the effects of NAC but not SNOAC, demonstrating the importance of eNOS in this process. NAC was also found to increase the DNA binding activity of the transcription factor hypoxia inducible factor-1 (HIF-1). This appears to be due, in part, by altering the interaction between HIF-1 and protein von Hippel Lindau via S-nitrosylation of cysteine 162. Together the data suggest that erythrocytic oxygen desaturation signals hypoxia through NO transfer reactions in vivo.

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(1) **Manuscript:** Palmer LA, Doctor A, Chhabra P, Sheram ML, Laubach VE, Karlinsky MZ, Forbes MS, Macdonald T, Gaston B. 2007 S-nitrosothiols signal hypoxia-mimetic vascular pathology J. Clin Investigation 117: (9) 2592-2601.

(2) **Abstract:** Palmer LA, Que L, Brown-Steinke K, de Ronde K, Gaston B. S-nitrosoglutathione Reductase, Gender, and the Development of Pulmonary Arterial Hypertension submitted to American Thoracic Society International Conference to be held in Toronto in May 2008

(3) **Submitted Grant** :Specific Aims of R21 National Institutes of Health Grant: Gender, GSNO-Reductase and the Development of Pulmonary Hypertension

Introduction:
Pulmonary arterial hypertension (PAH), high blood pressure within the lung, is a progressive disease which is characterized by an increase in pulmonary arterial pressure and the formation of muscle around normally non-muscular small pulmonary arteries. Without treatment, PAH progresses rapidly to right heart failure and death. The mechanism(s) sensing the initiating event and transducing this signal into changes in protein expression to alter pulmonary physiology are unclear. The role S-nitrosothiols (SNO) play in the development of PAH is examined in this research project. In the pulmonary circulation, erythrocytes deliver SNOs to recipient target proteins as a function of oxygen saturation. In this context, erythrocytes can act as a molecular switch, monitoring changes in oxygen saturation to deliver SNOs to the vascular endothelium. In the experiments performed, N-acetyl cysteine (NAC) is used as a tracer to 1) monitor SNO formation, transfer and metabolism *in vivo,* 2) address the physiological and pathological consequences of SNO signaling in the pulmonary vasculature*,* and 3) identify SNO target proteins in this signaling pathway *in vivo*. Differences in SNO formation, transfer and metabolism and the role this pathway plays in gender specific differences associated with the development of PAH are examined.

Body:

Completed Work
In vitro data suggest that nitric oxide (NO) transfer reactions (S-nitrosylations) occurring during erythrocyte deoxygenation may signal hypoxia (1-5). Studies for this grant period have focused on using the compound N-acetyl cysteine (NAC) as a tracer to examine the formation and transfer of SNOs from the blood to the pulmonary endothelium and the associated physiological/pathological consequences that may occur in the lung. Our studies demonstrate that chronic systemic treatment of NAC to C57BL6/129SvEv or C57Bl6 male mice result in the development of PAH as assessed by an increase in right heart weight, right ventricular pressure and muscularization of small pulmonary arterioles (Appendix, J Clin Invest manuscript Figure 1). This appears to be indistinguishable from that caused by chronic hypoxia. The pathological effects of NAC were both dose and time dependent. Low dose NAC (1mg/ml, 3 weeks) was ineffective in increasing the right ventricular weight but caused significant increases in right ventricular pressure (Table 1).

Table 1. Dose Dependent effects on Right Ventricular Weight and right Ventricular Pressure in NAC-Treated Male Mice

NAC Concentration	RV/LV +S	RVP (mm Hg)
untreated	0.252 + 0.0160	13.11 + 0.346
1 mg/ml	0.272	27.5
10 mg/ml	0.349 + 0.0326	18.06 + 0.651

In addition, the changes were time dependent; increases in right ventricular pressure occurred within one week (10mg/ml NAC) and persisted at 2-3 weeks (Table 2). On the

other hand, right ventricular weight increased more slowly, with significant differences only observed at the three week time point.

Table 2. **Time Course of changes in Right Ventricular Weight and Right Ventricular Pressure in 10mg/ml NAC Treated Male Mice**

	RV/VL+S	RVP (mm Hg)
untreated	0.252 + 0.0160	13.11 + 0.346
1 week	0.292 + 0.0326	22.80 + 3.30
3 weeks	0.349 + 0.326	18.06 + 0.651

Lastly, the pulmonary effects of concomitant 3 weeks exposure to both hypoxia and 10mg/ml NAC was not additive (Table 3), suggesting that there was functional overlap between the hypoxia and NAC- stimulated pathways.

Table 3. **Right Ventricular Weight and Right Ventricular pressures in male mice treated with both hypoxia and 10mg/ml NAC for 3 weeks**

	RV/LV +S	RVP (mm Hg)
Hypoxia	0.34 + 0.020	19.9 + 1.03
Hypoxia + NAC	0.35 + 0.082	21.4 + 1.30

The mechanism by which NAC may mimic the effects of hypoxia may be related to alterations in formation and/or transfer of SNOs from the blood to the endothelium and its delivery to target protein molecules. Published data have demonstrated NO in the form of nitrosonium (NO^+) is released from hemoglobin as a function of oxygen saturation (4, 6-8). Thus, the possibility exists that NO^+ released from the erythrocyte was transferred to NAC forming the S-nitrosylated derivative S-nitrosylated NAC (SNOAC) according the following reaction: protein-S-NO + NAC $\rightarrow$ protein-SH + SNOAC. To assess this possibility, total erythrocytic SNO concentration/hemoglobin (SNO_{RBC}/Hb) was measured in control and NAC-treated male mice using reductive chemiluminescence in the presence of carbon monoxide (4). NAC-treated mice had lower SNO_{RBC}/Hb content than control animals (Appendix, J Clin Invest manuscript figure 3A). Mass spectroscopy of plasma isolated from blood taken from the right ventricle of NAC-exposed animals demonstrated the presence of SNOAC (Appendix. J Clin Invest manuscript figure 3B). No SNOAC was seen in the control animals. This observation was confirmed by coelution with ^{15}N labeled SNOAC and by nitric oxide displacement from the S-NO bond using mercuric chloride (Appendix, J Clin Invest manuscript figure 3B). Ex vivo studies using human blood provided additional evidence for this concept: 1) increases in blood deoxygenation resulted in the formation of SNOAC in the serum (Appendix, J Clin Invest manuscript figure 3C) and 2) the loss of SNO_{RBC}/Hb and the increase of SNOAC were dependent on oxyhemoglobin saturation (Appendix, J Clin Invest manuscript figure 3D). The formation of SNOAC measured in this ex vivo system was pseudo-first-order in the excess of NAC and was relatively slow ($K\sim5.3 \times 10^{-10}$ M/s). There was no transfer from erythrocytes to NAC when blood was maintained at 100% saturation.

SNO formation is dependent on the activity of nitric oxide synthase isoforms (NOS) (9-12). Endothelial NOS is the primary NOS isoform found in endothelial cells and is proposed to be important in maintaining SNO_{RBC}/Hb content (13). To examine the importance of eNOS in the development of PAH in response to NAC, male eNOS deficient mice were examined. The SNO_{RBC}/Hb content of eNOS deficient mice was a log order lower that that seen in with type mice. At baseline, these mice had slight increases in right ventricular pressure and right ventricular weight relative to wild type background animals (C57Bl6). Surprisingly, these mice were completely protected form NAC-induced PAH (Appendix J Clin Invest manuscript figure 1A,B) suggesting that the chronic effects of NAC to cause PAH are eNOS dependent. However, they were not protected from SNOAC-induced PAH.

Proteins involved in the development of PAH have been identified using numerous animal models (14-20). Many of these proteins are also responsive to hypoxia. Therefore, the ability of NAC to regulate genes known to be modified by hypoxia and involved in PAH was examined in vivo. Whole lung expression of hypoxia-inducible mitogenic factor (HIMF) (21), fibronectin (22) and endothelial nitric oxide synthase (eNOS) (23-25) were increased both by hypoxia and 3 weeks of NAC treatment (Appendix, J Clin Invest manuscript figure 2A, B, C). In contrast, VEGF-A and endothelin protein did not increase significantly with hypoxia or NAC in our model system (Appendix, J Clin Invest manuscript 2E, F). It should be noted that coordinate increases in lung protein and mRNA were only observed for the expression of some genes known to be upregulated in hypoxia in vitro. Whole lung HIMF and eNOS mRNA expression were increased in response to NAC, although the latter increases were transient (Appendix, J Clin Invest manuscript figure 2 D,G) . Parallel increases in lung protein and mRNA were not observed for VEGF A (mRNA levels increased, no change in protein expression or fibronectin (a large increase in protein expression with little change in mRNA expression) (Appendix, J Clin Invest manuscript figure G). Taken together, this suggests that PAH associated vascular remodeling is more complex than would be predicted based on the effects of low pO_2 alone.

SNOs can have hypoxia-mimetic gene regulatory effects involving the expression of transcription factors relevant to pulmonary vascular disease. One such transcription factor is hypoxia inducible factor-1 (HIF-1). Expression and DNA binding activity of HIF-1 have been shown to be affected by SNOs in vitro (3, 26-28). We have demonstrated that HIF DNA binding activity is induced in the lungs of mice treated with NAC (Appendix, J Clin Invest manuscript figure 5A). The mechanisms by which SNOs alter HIF-1α abundance, however, are complex. NO transfer between thiols could stabilize HIF through effects on the prolyl hydroxylases, Akt signaling, and HIF-1 itself (26-31) Transnitrosation reactions have also been shown to modify the activity of E3 ubiquitin ligases, the enzymes necessary to specifically target proteins for ubiquitination and destruction (32). In vitro transcription studies indicate the S-nitrosylating agent S-nitrosoglutatione (GSNO) can alter HIF-1 ubiquitination (Appendix, J Clin Invest manuscript figure 5c). Furthermore, both SNOAC and GSNO inhibit the coimmunoprecipitation of HIF-1α with its E3 ligase, von Hippel Lindau protein (pVHL) in COS cells overexpressing both FLAG tagged-pVHL and HA-tagged HIF-1α (Appendix, J

Clin Invest manuscript figure 5D). Moreover, pVHL itself appears to be S-nitrosylated. (Appendix, J Clin Invest manuscript figure 5E, F). This appears to occur at residue C162 (Appendix, J Clin Invest manuscript figure 5G). This residue is required for pVHL to bind to elongin C and ubiquintate HIF-1α (33).   At this time there are several mechanisms by which S-nitrosylation may alter HIF-1 stability in normoxia. The extent to which each of these mechanisms is involved in the hypoxia mimetic vascular effects of SNOAC in vivo will need to be further explored.

Work in Progress:
S-nitrosothiol Bioavailablity and Gender
Differences in SNO bioavailability may be responsible for the gender discordance seen in the development of PAH. A comparison of data obtained from male and female C57BL6/129SvEV mice treated with NAC demonstrate female mice have higher right ventricular pressure (RVP) and right ventricular weight (RVW) than males at baseline, but are protected from the additional SNO-induced increases in RVP and RVW observed in males (Figure 1). Defining the mechanism(s) by which male mice are vulnerable to/female mice are protected from these SNO mediated changes may be instrumental in determining a cause for the gender discordance seen in the human form of the disease.
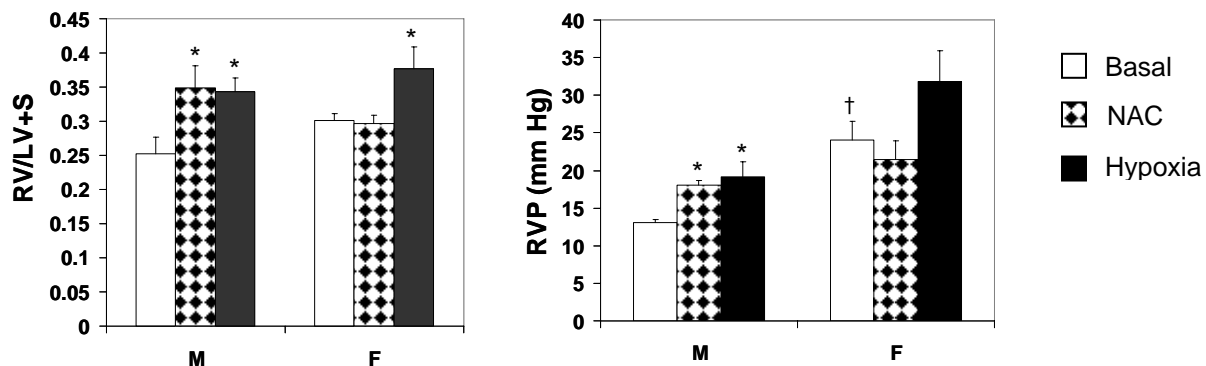

Figure 1. Right heart weight and right ventricle pressure is greater in female mice. Mice were untreated (N) or treated with 10mg/ml N-acetyl cysteine (NAC) or hypoxia (H) for a period of 3 weeks. (A) Right ventricular pressure (RVP), right heart weight (expressed as right ventricular weight/left ventricular weight + septum weight (RV/LV+S) were determined in male and female C57Bl6/129SvEv mice. Basal (untreated) right heart weight and RVP are greater in female animals. * Significant increases compared to basal levels p<0.05.   † Significant increases compared to male p<0.05.   N=20

Earlier data had demonstrated that NAC is S-nitrosylated forming SNOAC in the plasma (34). It should be noted that the levels of SNOAC found in the plasma of female animals is significantly less than SNOAC levels found in the plasma obtained from male animals (Figure 2).
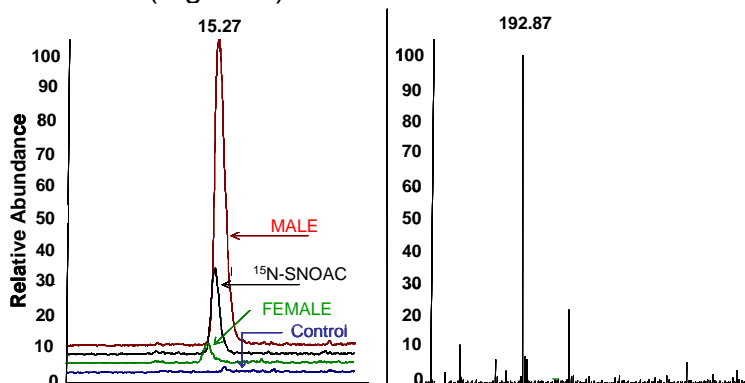

Figure 2. The amount of SNOAC formed in the serum of NAC treated mice is less in female animals. Serum SNOAC was measured by mass spectroscopy (MS) in C57BL6/129SvEv male and female mice treated with NAC.   Left panel = LC chromatogram: Right panel= MS spectrum. Serum from NAC-treated male (red) and female (green) mice had a SNOAC peak (m/z 193) that co-migrated with the ^{15}N-SNOAC standard (black). No signal was seen in non treated mice (blue). It should be noted that the amount of SNOAC detected was much less in the female mice.

The reason(s) for this difference is not known but could be explained by either 1) increased transfer of NO^+ from SNOAC to target proteins in the serum or on the endothelium or 2) altered transnitrosation equilibria with endogenously produced S-nitrosoglutathione (GSNO) though the actions of GSNO-reductase (GSNO-R).

To examine this latter possibility, GSNO-reductase expression and activity were measured in male and female mice. We hypothesized that the elevated eNOS expression in the lungs of female mice, due to the production of estrogen which is known to increase eNOS activity (35, 36), could result in greater SNO production/bioactivity compared to males. However, GSNO-reductase activity in female C57Bl6/129SvEv and C57BL6 mice was greater than that seen in males with no significant differences in protein expression (Figure 3). This increase in GSNO-reductase found in female animals could be postulated to equalize the SNO content between male and female animals by balancing the formation and degradation of SNOs.
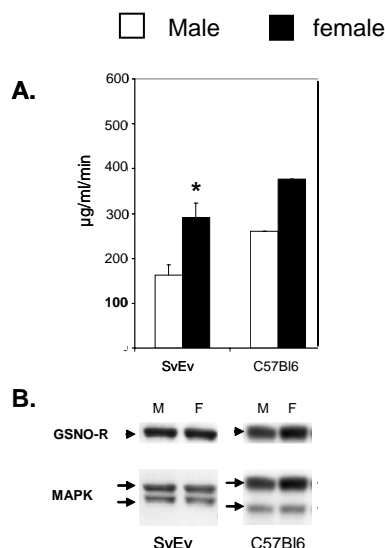

Figure 3. Activity of GSNO-reductase (GSNO-R) is greater in female mice. (A.) GSNO-R activity is measured in male and female C57Bl6/129SvEv (SvEV) and C57Bl6 mice. GSNO (35µM) was incubated with 500 ng/µl of lung homogenate supplemented with NADH (300 µM), GSH (2mM), ascorbic acid (500 µM), DTPA (100 µM) and PBS pH 7.4. After incubation at 37°C for 5 minutes the enzyme activity was quenched with trichloroacetic acid (5%) and GSNO determined by mass spectrometry. GSNO-R activity in the lung homogenates obtained from female mice was **elevated** compared to male mice in the C57Bl6/129SvEv (n=4) and C57Bl6 (n=2) animals. (B) Western blot demonstrating that protein expression of GSNO-R is similar in male and female mice of each strain of animal. Equal loading of the samples was determined by MAPK.

Analysis of total SNO levels in whole blood taken from the left ventricle of untreated male and female mice demonstrate no significant differences in SNO/Hb content (Table 4). The data, taken together, suggests that the production and the metabolism of endogenously produced SNOs are balanced in untreated animals.

Table 4. SNO/Hb levels are similar in untreated Male and Female C57Bl6/129SvEv Mice

Gender	SNO/Hb
Male	$7.75 \times 10^{-5} \pm 2.49 \times 10^{-5}$
Female	$8.75 \times 10^{-5} \pm 1.50 \times 10^{-5}$

Right ventricular blood was collected from male (n=4) and female (n=4) mice and subjected to reductive chemiluminescence in the presence of carbon monoxide (4). SNO values were normalized to hemoglobin content.

The development of PAH induced by NAC can be eliminated by castration (Figure 4). Moreover, castration also resulted in an increase in GSNO-reductase activity (reported in Figure 4 as the amount of GSNO remaining in the reaction). It should be noted that the GSNO-R activity was

similar to the level seen in female mice and thus may be related to the protection against NAC induced PAH.
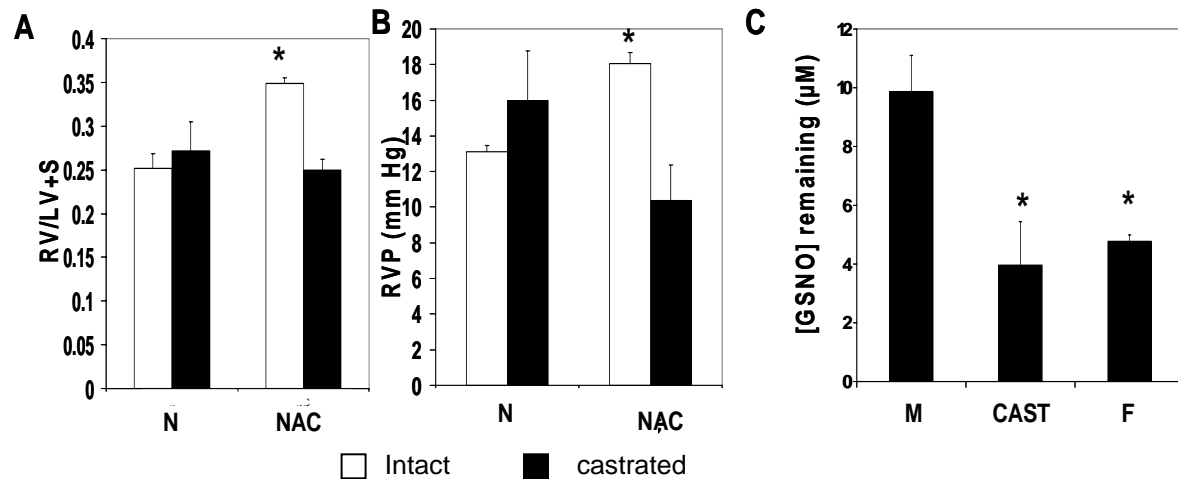

Figure 4. Castration eliminates the increase in right heart weight and right ventricular pressure induced by NAC as well as increases GSNO-reductase activity. Male C57BL6/129SvEv mice with either intact gonads or castrated were treated with or without NAC for 3 weeks. (A) Right heart weight and (B) right ventricular pressure were measured. Castration eliminated the increase in both right heart weight and right ventricular pressure (N=11 intact, N=10 castrated). (C) Castrated mice show an increase in GSNO-reductase activity compared to the male gonad intact animals. It should be noted that the levels of activity seen in the castrated mice is similar to that seen in the female mice. The data in C are presented as the amount of GSNO remaining after 5 min.

Endothelial/Erythrocyte Fibercell System

Work has begun on developing an in vitro model for erythrocyte/endothelial cell interactions using Fibercell technology. We have been able to successfully isolate mouse lung primary endothelial cells from our mice and can maintain the cells in culture for 10 passages (Appendix, J Clin Invest manuscript, Methods). Using these endothelial cells, we can demonstrate that endothelial cells grown under shear force have an increase in eNOS expression (Figure 5) as previously reported (37, 38).
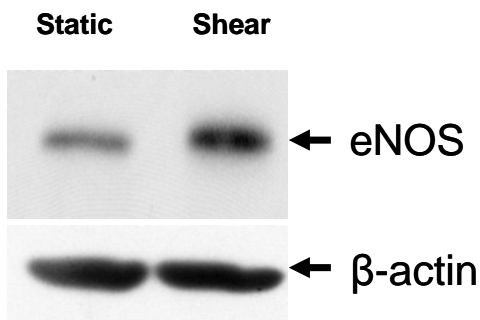

Figure 5. Mouse Lung Endothelial Cells show increased endothelial Nitric Oxide Synthase (eNOS) under shear stress. Mouse lung endothelial cells were seeded onto fibers 24 hours prior to applying shear stress (15 dynes/cm^2) for 48 hours. Endothelial nitric oxide synthase protein levels within cell lysates obtained from cells grown under stress were compared to those grown under static conditions.

This experiment demonstrates that we can grow these cells in culture on fibers, subject them to shear force, and obtain cell lysate for analysis. Our challenge now is to develop a reproducible way to subject the cells to deoxygenation in the absence and presence of erythrocytes. Experiments in progress include 1) exposure to hypoxia and 2) creating

an oxygen gradient within the length of fibers. Present experiments have focused on the measurement and reproducibility of creating hypoxia within this system in the presence of tissue culture media. Problems with adequate deoxygenation of the media within this system are being solved through the use of a hypoxic work station.

Key Research Accomplishments:

Specific Aim 1: Aberrant formation/transfer and/or delivery of S-nitrosothiols (SNOs) leads to the development of Pulmonary Hypertension

Task 1: Characterize SNO formation/transfer/metabolism in vivo and in vitro in male animals following chronic N-acetyl cysteine administration.
a. Characterize the in vivo conversion of NAC to SNAOC by measuring SNOAC levels in the plasma. J. Clin Investigation 117: (9) 2592-2601, 2007; Figure 3b

b. Measure rate of formation of SNOAc as a function of dose, time, and red blood cell deoxygenation. J. Clin Investigation 117: (9) 2592-2601, 2007; Figures 3c, 3d.

c. Determine SNO-hemoglobin levels present in blood J. Clin Investigation 117: (9) 2592-2601, 2007; Figure 3A

d. Measure the abundance and activity of the GSNO-degradation enzyme S-nitrosoglutathione reductase (GSNO-R) (Figure 3 in text)

Task 2: Identify the pathophysiological changes caused by NAC/SNOAC treatment in the pulmonary vasculature in vivo.

a. Measure changes in right heart weight, right ventricular pressure, and vascular muscularization to assess the development of pulmonary hypertension. J. Clin Investigation 117: (9) 2592-2601, 2007; Figure 1a-e.

b. Evaluate changes in NAC/SNOAC induced pulmonary hypertension as a function of treatment time, dose (Tables 1-3 in text) and reversibility.

c. Measure right heart weight, right ventricular pressure, and vascular muscularization in wild type mice and eNOS knockout mice J. Clin Investigation 117: (9) 2592-2601, 2007; Figure 1a,b

d. Set up a physiological relevant in vitro endothelial culture system using "Fibercell" technology to establish normal flow mediated shear stress.
    a. Isolate primary mouse pulmonary vascular endothelial cells. (J. Clin Investigation 117: (9) 2592-2601, 2007, methods)
        b. Confirm shear stress alters eNOS expression. (Figure 5 in text.)

Specific Aim 2. NAC/SNOAC signaling alters hypoxia inducible factor (HIF) expression and activity

Task 1: Determine if NAC/SNOAC alters HIF expression and activity

a. Evaluate HIF-1α ubiquitination in control and NAC/SNOAC treated mice to evaluate HIF protein degradation. J. Clin Investigation 117: (9) 2592-2601, 2007, Figure 5c

b. Quantify HIF-1 and HIF-1 activity by measuring DNA Binding. (J. Clin Investigation 117: (9) 2592-2601, 2007 Figure 5a,b)

Task 2.   Determine if NAC/SNOAC modifies the expression of HIF-1 responsive genes implicated in the development of PH.

Measure expression levels of VEGF, ET-1, HIMF, eNOS, Fibronectin; J. Clin Investigation 117: (9) 2592-2601, 2007 Figure 2a-g

Specific Aim 3.   Gender differences in pulmonary hypertension arise from an imbalance of SNO formation and metabolism.

Task 1.   Measure the pathophysiological parameters consistent with pulmonary hypertension following chronic administration of NAC/SNOAC to female animals for comparison to that measured in males. (Figure 1 in text)

Task 2.   Identify differences in SNOC formation/transfer/metabolism between male and female animals.

Measure SNO formation/transfer/metabolism (Table 4 and Figure 2 in text).

Reportable Outcomes.
Manuscripts:
Palmer LA, Doctor A, Chhabra P, Sheram ML, Laubach VE, Karlinsky MZ, Forbes MS, Macdonald T, Gaston B. S-nitrosothiols signal hypoxia-mimetic vascular pathology J. Clin Investigation 117: (9) 2592-2601, 2007.

Abstracts:
Palmer LA, Que L, Brown-Steinke K, de Ronde K, Gaston B. S-nitrosoglutathione Reductase, Gender, and the Development of Pulmonary Arterial Hypertension submitted to American Thoracic Society International Conference to be held in Toronto in May 2008

New Grant applications:
R21 National Institutes of Health: Gender, GSNO-Reductase and the Development of Pulmonary Hypertension: submitted October 2007 for Review in February 2008.

Conclusions
In vitro evidence suggest that S-nitrosylation reactions that occur during erythrocyte deoxygenation may signal hypoxia. The studies published in the Journal of Clinical

Investigation indicate that this process may also be important in vivo. These studies provide 1) the first in vivo measurements suggesting that NO can signal erythrocyte oxygen desaturation through the formation of SNOs; (2) introduce a novel murine model of PAH; (3) provide at least one explanation for the previously reported hypoxia-mimetic effects of NAC and (4) suggest that PAH may be a risk of systemic therapy with NAC.

Women are more likely than men to develop PAH (39, 40). However, the molecular basis for this gender difference is not known. In our animal model, chronic administration of N-acetyl cysteine (NAC) elicits PAH indistinguishable from that induced by chronic hypoxia in males (34). This effect of NAC is not seen in females (Figure 1). Moreover, serum levels of SNOAC responsible for initiating the NAC-induced PAH (34), are substantially less in females than in males (Figure 2). Consistent with this observation, GSNO-reductase activity is greater in female than in males (Figure 3) with no change in protein expression. Moreover, castration prevented the development of NAC-induced PAH in male animals. Figure 5) which could be a result of the observed increase in GSNO-reductase activity in the castrated males to the level seen in females (Figure 4). Taken together, the results suggest that GSNO-reductase may be responsible for murine male susceptibility/female protection from the development of PAH induced by exogenous elevation of SNOs and that testosterone may play a role.

References

1. Lipton AJ et al. 2001 S-Nitrosothiols signal the ventilatory response to hypoxia. Nature 413:171-74.
2. Jia L et al. 1996 S-nitrosohaemoglobin: a dynamic activity of blood involved in vascular control Nature 380:221-226;
3. Palmer LA, Gaston B, Johns RA 2000 Normoxic stabilization of hypoxia-inducible factor-1 expression and activity: Redox dependent effect of nitrogen oxides Mol Pharmacol 58: 1197-1203;
4. Doctor A. et al. 2005 Hemoglobin conformation couples erythrocyte S-nitrosothiol content to O_2 gradients. Proc Natl Acad Sci U.S. A. 102: 5709-5714;
5. Pawloski JR, Hess DT, Stamler JS 2001 Export by red blood cells of nitric oxide bioactivity. Nature 409: 622-626.
6. Singel DJ and Stamler JS. 2005 Chemical Physiology of blood flow regulation by red blood cells: The role of nitric oxide and S-nitrosohemoglobin. Annu. Rev. Physiol. 67: 99-145.
7. Stamler J.S. et al. 1997 Blood flow regulation by S-nitrosohemoglobin in the physiological oxygen gradient. Science 276: 2034-2037.
8. Sonveaux P et al. 2007 Transport and peripheral bioactivities of nitrogen oxides carried by red blood cell hemoglobin: role in oxygen delivery Physiology. 22:97-112.
9. Gow, A. J. et al. 2002 Basal and stimulated protein S-nitrosylation in multiple cell types and tissues. J. Biol. Chem. 277: 9637-9640.
10. Mayer, B. et al. 1998 A new pathway of nitric oxide/cyclic GMP signaling involving S-nitrosoglutathione. J. Biol. Chem. 273: 3264-3270.

11. Jaffrey SR. et al. 2001 Protein S-nitrosylation: a physiological signal for neuronal nitric oxide. Nature Cell Biol 3: 193-97.
12. Eu JP, Liu L, Zeng M, Stamler JS. 2000 An apoptotic model for nitrosative stress. Biochemistry 39: 1040-1047.
13. McMahon TJ et al 2005. A nitric oxide processing defect of red blood cells created by hypoxia: deficiency of S-nitrosohemoglobin in pulmonary hypertension. Proc. Natl Acad. Sci. U.S. A. 102: 14801-14806.
14. Steudel W. et al. 1977 Pulmonary vasoconstriction and hypertension in mice with targeted disruption of the endothelial nitric oxide synthase (NOS3) gene. Circ Res. 81, 34-41.
15. Yu A. Y. et al. 1999 Impaired physiological responses to chronic hypoxia in mice partially deficient for hypoxia - inducible factor 1α. J. Clin Invest 103: 691-696.
16. Brusselmans K. et al. 2003 Heterozygous deficiency of hypoxia-inducible factor-2α protects mice against pulmonary hypertension and right ventricular dysfunction during prolonged hypoxia. J. Clin Invest 11:1519-1527.
17. Geraci M.W. et al. 1999 Pulmonary prostacyclin synthase overexpression in transgenic mice protects against development of hypoxia pulmonary hypertension. J. Clin Invest 103: 1509-1515.
18. Eddahibi S. et al 2000 Attenuated hypoxia pulmonary hypertension in mice lacking the 5' hydroxytryptamine transporter gene. J. Clin Invest 105: 1555-1562.
19. Campbell, AI, Zhao Y, Sandhu, R Stewart DJ. 2001 Cell-based gene transfer of vascular endothelial growth factor attenuates monocrotaline-induced pulmonary hypertension. Circulation 104: 2242-2248.
20. Carmeliet P. and Collen D. 1998 Vascular development and disorders: molecular analysis and pathogenic insights. Kidney Int. 53: 1519-1549.
21. Teng X, Li D. Champion HC and Johns RA 2003 FIZZ1.RELMalpha, a novel hypoxia-induced mitogenic factor in lung with vasoconstrictive and angiogenic properties Cir Res 92: 1065-1067
22. Vyas-Somani A.C. et al 1996 Temporal alteration in basement membrane components in the pulmonary vasculature of the chronically hypoxic rat: impact of hypoxia and recovery. Am J Med Sci 312: 54-67.
23. Le Cras T et al 1999 Effect of chronic hypoxia and altered hemodynamics on endothelial nitric oxide synthase expression in the adult rat lung. J. Clin. Invest. 101:795-801.
24. Lam C-F, Peterson TE, Croatt AJ, Nath KA and Katusic ZS 2005 Functional adaptation and remodeling of pulmonary artery in flow induced pulmonary hypertension. Am. J. Physiol Heart Circ Physiol. 289: H2334-H2341.
25. Zue C and Johns RA 1996 Upregulation of nitric oxide synthase correlates temporally with the onset of pulmonary vascular remodeling in the hypoxia rat. Hypertension 28: 743-753.
26. Sandau KB, Faus HG and Brune B 2000 Induction of hypoxia-inducible factor-1 by nitric oxide is mediated via the PI3K pathway. Biochem Biophys Res Com 278:263-267.

27. Sunbayev VV, Budde A, Zhou J and Brune B 2003   HIF-1 alpha protein as a target for S-nitrosation FEBS Lett <u>535</u>: 106-112.
28. Li F et al. 2007 Regulation of HIF-1α stability through S-nitrosylation. Mol Cell 26: 63-74.
29. Ahow J and Brune B 2005 NO and transcripton regulation: from signaling to death. Toxicology <u>208</u>:223-233.
30. Metzen E. et al. 2003 Nitric oxide impairs normoxic degration of HIF-1α by inhibition of prolyl hydroxylases. Mol Biol Cell <u>14</u>: 3437-3481.
31. Kohl R Ahou J and Brune B 2006 Reactive oxygen species attenuate nitric oxide mediated hypoxia inducible factor 1 alpha stabilization. Free Radic Biol Med <u>40</u>: 1430-1442.
32. Yao D. et al. 2004 Nitrosative stress linked to sporadic parkinsons's disease: S-nitrosylation of Parkin regulates its E3 ligase activity Proc Natl Acad Sci U.S. A. <u>101</u>: 10810-10814.
33. Ohh M. et al. 2000. Ubiquitination of hypoxia inducible factor requires direct binding to the beta domain of the von Hippel-Lindau protein. Nat Cell Biol <u>2</u>: 423-427.
34. Palmer L. A. et. al. 2007 S-nitrosothiols signal hypoxia-mimetic vascular pathology. J. Clin. Invest. <u>117</u>: 2592-2601.
35. Chen Z. et al. 1999 Estrogen receptor alpha mediates nongenomic activation of eNOS by estrogen. J. Clin Invest <u>103</u>: 401-6.
36. Goetz R. M. et al. 1999. Estradiol induces the calcium-dependent translocation of endothelial nitric oxide synthase. Proc Natl Acad Sci USA <u>96</u>: 2788-2793.
37. Toda M. et al. 2008 Differential gene responses in endothelial cells exposed to a combination of shear stress and cyclic stress. J. Biotechnol. <u>133</u>: 2390-244.
38. Searles CD. 2006 Transcriptional and posttranscriptional regulation of endothelial nitric oxide synthase expression. Am J. Physiol Cell Physiol 291: C803-816.
39. Runo JR and Loyd JE. 2003 Primary pulmonary hypertension. *The Lancet* <u>361</u>: 1533-44.
40. Gaine S.P. and Rubin J.L. 1998 Primary Pulmonary Hypertension <u>352</u>:719-725.

Appendices

(**1**) Manuscript: Palmer LA, Doctor A, Chhabra P, Sheram ML, Laubach VE, Karlinsky MZ, Forbes MS, Macdonald T, Gaston B. S-nitrosothiols signal hypoxia-mimetic vascular pathology   J. Clin Investigation <u>117</u>: (9) 2592-2601, 2007

(**2**) Abstract: Palmer LA, Que L, Brown-Steinke K, de Ronde K, Gaston B. S-nitrosoglutathione Reductase, Gender, and the Development of Pulmonary Arterial Hypertension submitted to American Thoracic Society International Conference to be held in Toronto in May 2008

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S-Nitrosothiols signal hypoxia-mimetic vascular pathology

Lisa A. Palmer,[1] Allan Doctor,[1] Preeti Chhabra,[1] Mary Lynn Sheram,[1] Victor E. Laubach,[2] Molly Z. Karlinsey,[3] Michael S. Forbes,[1] Timothy Macdonald,[3] and Benjamin Gaston[1]

[1]Department of Pediatrics and [2]Department of Surgery, University of Virginia School of Medicine, and [3]Department of Chemistry, University of Virginia, Charlottesville, Virginia, USA.

NO transfer reactions between protein and peptide cysteines have been proposed to represent regulated signaling processes. We used the pharmaceutical antioxidant *N*-acetylcysteine (NAC) as a bait reactant to measure NO transfer reactions in blood and to study the vascular effects of these reactions in vivo. NAC was converted to *S*-nitroso-*N*-acetylcysteine (SNOAC), decreasing erythrocytic *S*-nitrosothiol content, both during whole-blood deoxygenation ex vivo and during a 3-week protocol in which mice received high-dose NAC in vivo. Strikingly, the NAC-treated mice developed pulmonary arterial hypertension (PAH) that mimicked the effects of chronic hypoxia. Moreover, systemic SNOAC administration recapitulated effects of both NAC and hypoxia. eNOS-deficient mice were protected from the effects of NAC but not SNOAC, suggesting that conversion of NAC to SNOAC was necessary for the development of PAH. These data reveal an unanticipated adverse effect of chronic NAC administration and introduce a new animal model of PAH. Moreover, evidence that conversion of NAC to SNOAC during blood deoxygenation is necessary for the development of PAH in this model challenges conventional views of oxygen sensing and of NO signaling.

Introduction

NO transfer reactions between protein and peptide cysteines have been proposed to represent regulated signaling processes (1, 2). For example, NO transfer from deoxygenated erythrocytes to glutathione ex vivo forms *S*-nitrosoglutathione (GSNO) (3). GSNO can signal acute vascular and central ventilatory effects characteristic of oxyhemoglobin desaturation (3–4) that are regulated by γ-glutamyl transpeptidase (GGT), GSNO reductase (GSNOR), and other enzymes (1, 3–6). However, direct measurement of *S*-nitrosothiol signaling in vivo has proven challenging because of the metabolism and tissue-specific localization of endogenous *S*-nitrosothiol species (1, 3, 4, 6). We have addressed these challenges by using *N*-acetylcysteine (NAC) as a bait reactant, allowing the stable NO transfer product, *S*-nitroso-*N*-acetylcysteine (SNOAC), to be distinguished by mass spectrometry (MS) from endogenous *S*-nitrosothiols. We report that NAC is converted to SNOAC in mice in vivo. Furthermore, chronic, systemic administration of either NAC or SNOAC to mice causes hypoxia-mimetic pulmonary arterial hypertension (PAH). These data reveal a previously unappreciated vascular toxicity of NAC and of *S*-nitrosothiols. Moreover, they suggest that *S*-nitrosothiol transfer reactions can signal hypoxia in vivo.

PAH is characterized by increased pressure in the pulmonary arteries (PAs), increased RV weight, and thickening and remodeling of small PAs. Untreated human PAH can progress to right heart failure and death (7, 8). Chronic hypoxia can cause PAH, and hypoxia-regulated genes in the pulmonary endothelium play a role (7, 9). The partial pressure of O_2 (pO_2) of blood in the hypoxic PA is not as low as that used to study the expression of hypoxia-modified genes in vitro (10). However, endothelium-derived NO in erythrocytes can be transferred as a nitrosonium (NO^+) equivalent to cysteine thiolates on glutathione (GSH) and anion exchange protein 1 (AE1) — forming GSNO and *S*-nitroso-AE1 — during erythrocytic oxyhemoglobin desaturation in vitro; this effect is dependent on erythrocytic oxyhemoglobin saturation rather than pO_2 (3, 4, 11, 12). *S*-Nitrosothiols, in turn, can increase the expression of hypoxia-associated gene regulatory proteins in vitro (5, 13, 14), an effect regulated by *S*-nitrosothiol metabolic enzymes (5, 14). Here, we report a hypoxia-mimetic effect, pulmonary vascular remodeling, that is associated with chronic systemic conversion of NAC to SNOAC in vivo. Strikingly, chronic exogenous administration of SNOAC — a vasodilator (15) — recapitulates the effects of hypoxia on the lung, causing hypoxia-mimetic murine PAH. Furthermore, experiments in eNOS-deficient mice reveal that eNOS is necessary for NAC, but not SNOAC, to cause PAH. Taken together, these data demonstrate an unanticipated potential toxicity of chronic, systemic NAC and SNOAC administration and suggest a novel paradigm for understanding hypoxia-associated disease.
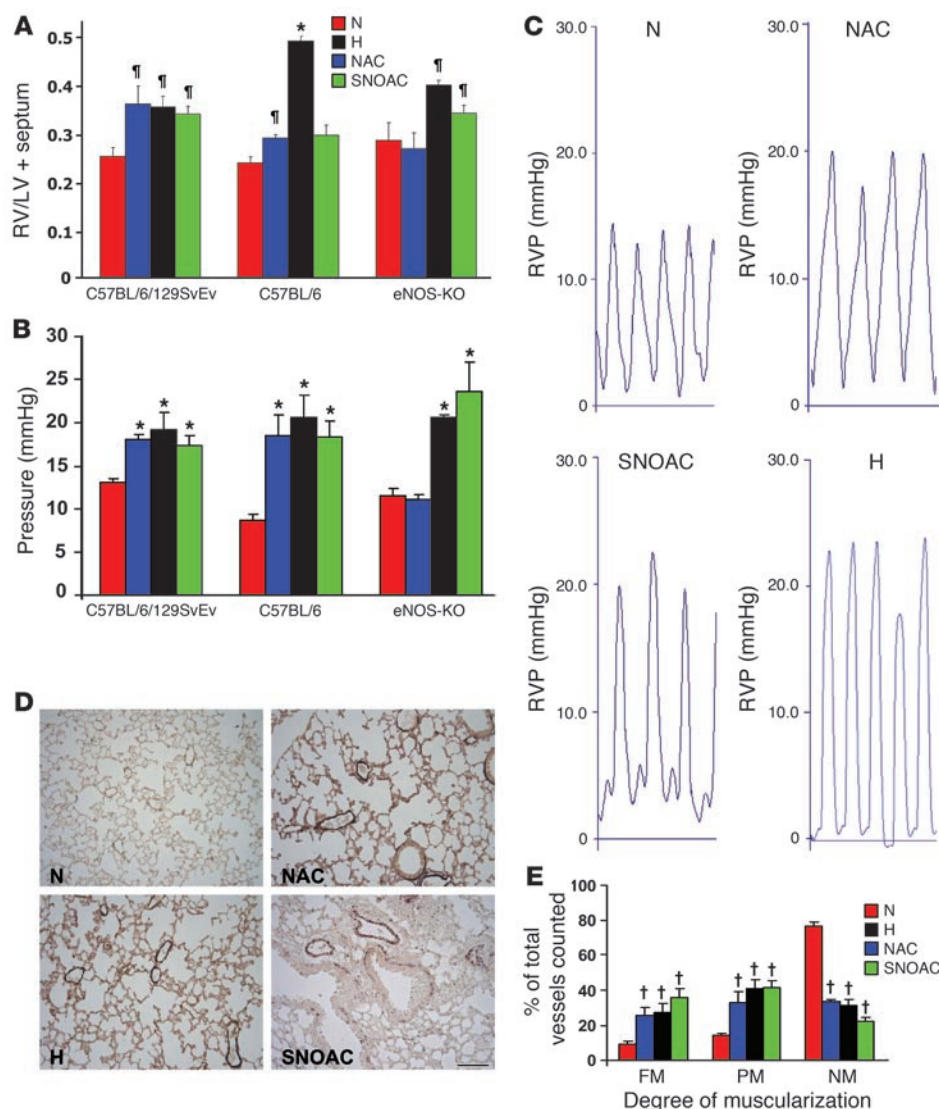
Results

NAC and SNOAC cause hypoxia-mimetic PAH. Three weeks of normobaric hypoxia (10%) increased RV systolic pressure, RV weight (relative to LV and septum [LV+S]) and muscularization of small (<80-μm) pulmonary arterioles in both C57BL/6 and C57BL/6/129SvEv mice (n = 4–14 each; Figure 1, A–E). The effects of 3 weeks' exposure to either NAC (10 mg/ml [52 mM]) or SNOAC (1 mM) in the drinking water were similar to those of 3 weeks' exposure to hypoxia in C57BL/6/129SvEv mice, increasing RV pressure (n = 3–4 each, $P < 0.001$ relative to normoxia), RV/LV+S weight ratio (n = 6–35 each; $P < 0.02$ relative to normoxia), and muscularization of the small pul-

Figure 1
Systemic NAC and SNOAC cause hypoxia-mimetic PAH in mice. C57BL/6/129SvEv, C57BL/6, and eNOS^{−/−} male mice were maintained in normoxia (N, red; 21% O_2) or hypoxia (H, black; 10% O_2); or were treated with NAC (blue) or SNOAC (green) in their drinking water for 3 weeks. (**A**) Relative RV weight was determined as the ratio of the weight of the RV to the LV+S weight. (**B**) RV systolic pressures were measured in the closed chest using a Millar 1.4 F catheter/transducer. (**C**) Representative RV pressure (RVP) tracings (each = 1 s). (**D**) Lung section images from C57BL/6/129SvEv mice immunostained for von Willebrand factor and α-SMA to illustrate changes in muscularization after 3 weeks of exposure to normoxia, hypoxia, NAC, or SNOAC. Scale bar: 100 μm (applies to all panels). (**E**) Changes in muscularization in C57BL/6/129SvEv mice in the small (<80-μm) vessels from histological sections (as in **D**) counted by an observer blinded to the protocol. FM, fully muscular; PM, partly muscular, NM, nonmuscular. Significant increases in muscularization in each treatment group were seen in comparison to normoxic controls. Data are mean ± SEM. ¶P < 0.02, *P < 0.001, †P < 0.003, by 1-way ANOVA followed by pairwise comparison, all compared with normoxic control.

monary arterioles. (n = 3–8; P < 0.003 relative to normoxia) (Figure 1, A–E). Of note, PA pressures in the treatment groups were greater than 125% those in controls in the hypoxia, NAC, and SNOAC groups, despite anesthesia, which can blunt PAH (ref. 8; Figure 1C). Similar results were obtained in C57BL/6 mice (n = 4–12 each); however, the increase in RV/LV+S ratio was not significant in these mice in the case of SNOAC (Figure 1, A and B).

In dose-response experiments, higher-dose NAC (10 mg/ml) increased RV weight relative to normoxia at 3 weeks in C57BL/6/129SvEv mice (Figure 1A); however, at a lower dose (1 mg/ml), RV/LV+S was normal at 3 weeks (0.27 ± 0.005; n = 5; P = NS compared with no treatment). On the other hand, low-dose NAC increased RV systolic pressure at 3 weeks to 27.5 mmHg (n = 4; P < 0.04 compared with normoxia).

In time course experiments performed using C57BL/6/129SvEv mice, there was a significant increase in RV pressure after 1 week of NAC exposure (10 mg/ml; mean RV pressure with NAC, 22.8 ± 3.3 mmHg, versus without NAC, 13.7 ± 0.7 mmHg; n = 5–8 each; P < 0.01), persisting at 2 and 3 weeks. RV/LV+S increased more slowly: a significant difference between NAC-

exposed and -unexposed animals was not observed until 3 weeks (n = 5–8 each at 1 and 2 weeks; P = NS). Concomitant 3-week exposure to both hypoxia and 10 mg/ml NAC did not result in a greater increase in mean RV/LV+S (0.35 ± 0.082 after NAC and hypoxia together versus 0.34 ± 0.020 after hypoxia alone; n = 4–6 each; P = NS) or RV pressure (21.4 ± 1.30 mmHg after NAC plus hypoxia versus 19.9 ± 1.03 mmHg after hypoxia alone; n = 4–6 each; P = NS), suggesting functional overlap between hypoxia- and NAC-stimulated pathways.

Whole-lung expression of hypoxia-inducible mitogenic factor (HIMF), fibronectin, and eNOS, proteins associated with the development of PAH in some models (16–20), was increased by hypoxia and by 3 weeks of NAC treatment (10 mg/ml; n = 3–6 each; P < 0.05 [HIMF and fibronectin] and P < 0.01 [eNOS]; Figure 2, A–D). Of note, VEGF-A and endothelin 1 protein expression did not increase significantly with hypoxia or NAC in our model (Figure 2, E and F). Though these proteins can be upregulated by low pO_2 in vitro, results are variable in PAH models (21–26), suggesting that PAH-associated vascular remodeling is more complex than would be predicted based on the effects of low pO_2 alone. Indeed, VEGF and
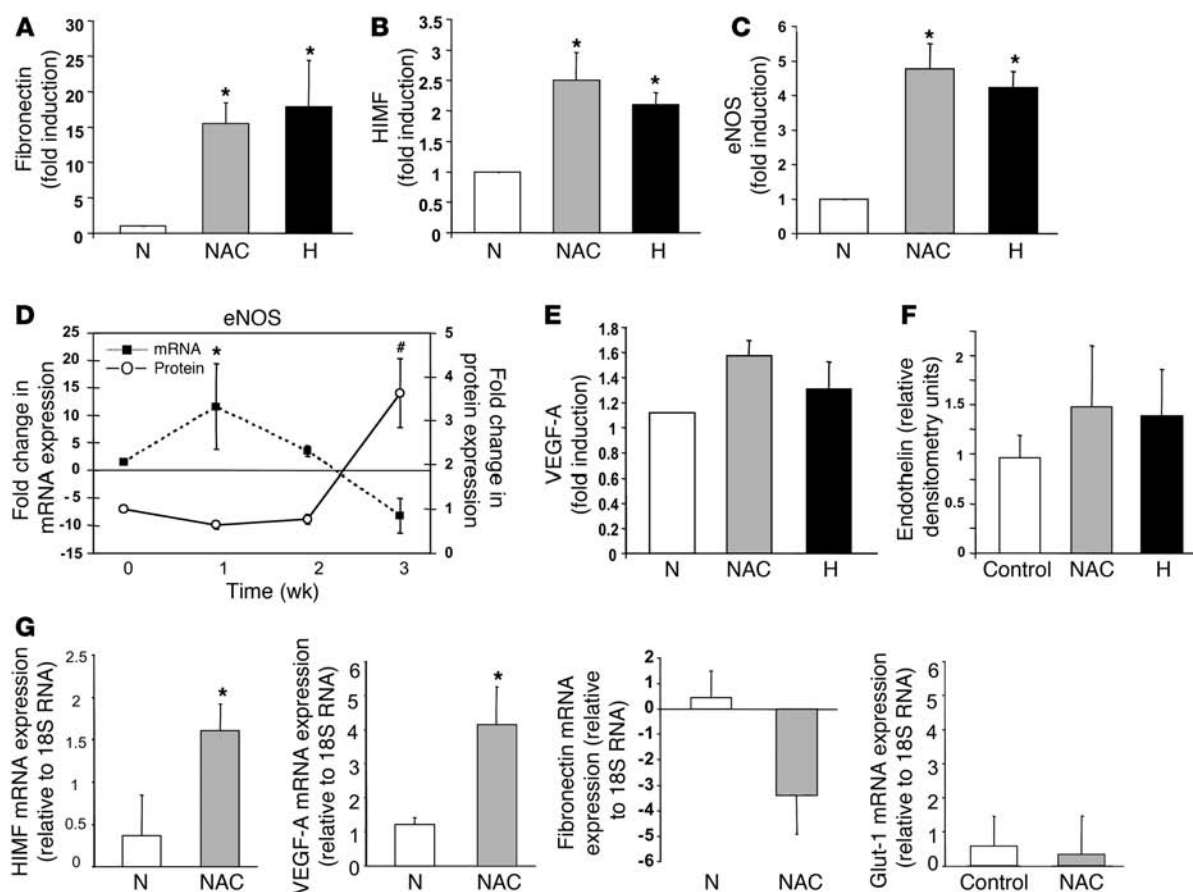
Figure 2
Three weeks of NAC treatment or hypoxia increases the whole-lung expression of certain genes associated with the development of PAH in mice. The expression of fibronectin (**A**), HIMF (**B**), eNOS (**C** and **D**), VEGF-A (**E**), and endothelin (**F**) in whole-lung homogenates from NAC-treated mice was examined by immunoblot. Fold increase in density relative to MAPK (equal loading control) was determined for each condition. The increases in fibronectin, HIMF, and eNOS (n = 3–5 each) were significant. (**G**) Three weeks of NAC treatment also increased whole-lung mRNA, assayed relative to 18S RNA by RT-PCR, for *HIMF* and *VEGF-A* but not *fibronectin* or *Glut-1* (n = 3 each). Time course analysis of NAC-treated mice (**D**) revealed that the increase in whole-lung *eNOS* mRNA (filled squares, left axis) preceded the increase in eNOS protein expression (open circles, right axis) but decreased by 3 weeks. $*P < 0.05$; $\#P < 0.01$.

eNOS expression can be associated with both protection against PAH and development of PAH, depending on the model and time course (18–29). NAC did not change expression of neuronal or inducible NOS isoforms at 3 weeks (data not shown). NAC exposure increased whole-lung *HIMF* mRNA expression and transiently increased *eNOS* mRNA (Figure 2, D and G). Interestingly, parallel increases in lung protein and mRNA were not observed for the expression of some genes known to be upregulated in hypoxia in vitro, either because of nonvascular expression measured in whole-lung homogenates and/or because of the complexity of pathways involved in the response to hypoxia and the development of PAH in vivo. For example, mRNA levels for *VEGF-A* increased after 3 weeks of NAC treatment (n = 3; P < 0.04; Figure 2G), though there was no significant change in VEGF-A protein levels (Figure 2E). On the other hand, the change in mRNA expression for *fibronectin* was not significant (n = 2–5 each; P = NS; Figure 2G), though expression of the corresponding protein increased (described above; Figure 2A). Further, mRNA for glucose transport protein–1 (*Glut-1*), conventionally upregulated by hypoxia, did not increase (n = 3; P = NS). Consistent with this complexity, our time course analysis

revealed an NAC-induced increase in whole-lung *eNOS* mRNA that preceded the NAC-induced increase in eNOS protein expression but was transient (Figure 2D).

Three weeks of NAC (10 mg/ml) did not affect systemic or portal vascular morphology (Supplemental Data; supplemental material available online with this article; doi:10.1172/JCI29444DS1) or hemoglobin (Hb) (12.5 ± 0.64 g/dl after NAC treatment versus 12.1 ± 0.65 g/dl in controls). Likewise, 3 weeks of oral SNOAC (1 mM) did not affect the systemic or portal vasculature (Supplemental Data).

NAC is converted to SNOAC in vivo and during erythrocytic deoxygenation in vitro and in vivo. The chemical mechanisms, including both inhibition of nitrosative/oxidative stress (30–32) and NO transfer chemistry (1, 33), by which NAC could cause PAH were investigated. NAC did not affect pulmonary vascular immunostaining for 3-nitrotyrosine (Supplemental Data), suggesting that its ability to cause PAH was not simply the result of tissue injury associated with altered nitrosative or oxidative stress.

In contrast, the ratio of total erythrocytic *S*-nitrosothiol concentration to Hb (SNO$_{rbc}$) (11) in NAC-treated mice (1.0×10^{-4} ±
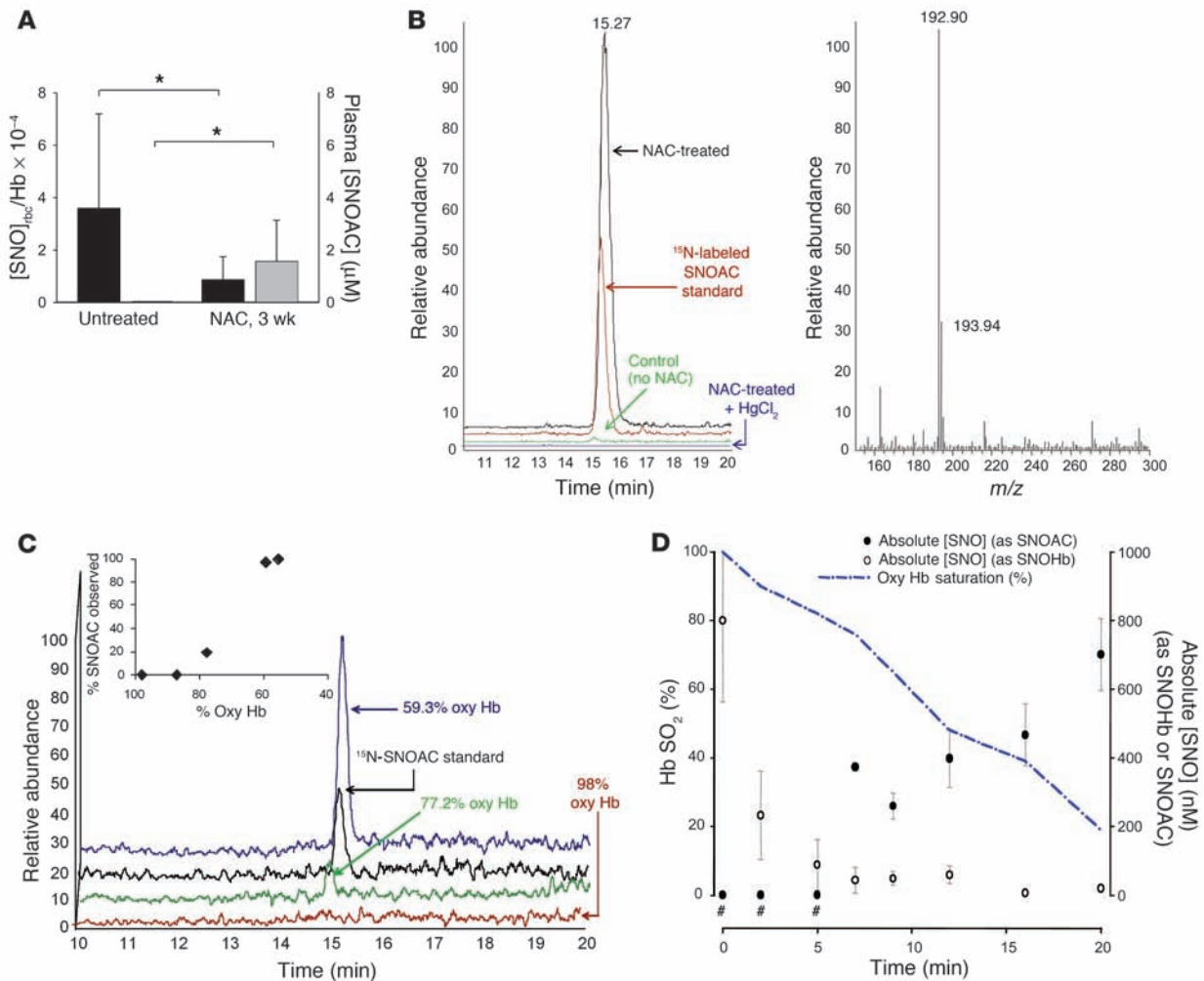
Figure 3
SNOAC is formed from NAC in blood ex vivo and in vivo. (**A**) The SNO_{rbc} in heparinized LV blood (black bars), measured by reductive chemilu-minescence (11), was lower than normal following 3 weeks of treatment with 10 mg/ml NAC ($n = 3$–4 each). In the same mice, plasma SNOAC levels (gray bars; measured by MS) increased from undetectable to approximately 2 μM over the same time (*$P < 0.05$). (**B**) Serum SNOAC, measured by MS, formed in NAC-treated mice (3 weeks). Left: liquid chromatogram; right: MS spectrum. NAC-treated mice had a SNOAC peak (m/z 193; red) coeluting with ^{15}N-labeled SNOAC standard (m/z 194; black) that was absent in untreated animals (green) and was not detected in NAC-treated mice after serum pretreatment with $HgCl_2$ to displace NO^+ from the thiolate (blue). (**C**) Oxygenated erythrocytes were deoxygenated ex vivo (argon; ref. 11) in the presence of 100 μM NAC; supernatant SNOAC was measured by MS (above). SNOAC concentration increased with oxyhemoglobin (Oxy Hb) desaturation (co-oximetry: inset), being maximal at 59.3% saturation (blue), less at 77.2% saturation (green), and undetectable at 98% saturation. (**D**) SNOAC (filled circles) accumulated as the concentration of S-nitrosothiol–modified Hb (SNOHb; open circles) and oxyhemoglobin saturation (Hb SO_2; blue line) both decreased in heparinized whole blood using argon with 5% CO_2 (pH 7.3) in a tonometer. Both the increase in SNOAC and the loss of SNO_{rbc} between 0 and 20 minutes were significant ($P < 0.01$ by ANOVA followed by pairwise comparison to the maximum value; $n = 3$). #SNOAC levels were below the limit of detection when the oxyhemoglobin saturation was greater than 80%.

0.7×10^{-4}; $n = 4$) was lower than that in control animals (3.7×10^{-4} $\pm 3.2 \times 10^{-4}$; $n = 3$; $P < 0.05$; Figure 3A). Decreased SNO_{rbc} content in NAC-treated mice could be expected if the NO group on eryth-rocytic protein thiols were transferred (1, 33) to NAC according to the reaction: protein-S-NO + NAC → protein-SH + SNOAC. There-fore, the formation of plasma SNOAC was assayed by MS. SNOAC was identified in the RV plasma of the NAC-exposed animals (1.6 ± 0.9 μM versus 0 μM in unexposed animals; $P = 0.04$; Fig-ure 3A), a finding confirmed both by coelution with ^{15}N-labeled SNOAC and by NO displacement from the S-nitrosothiol bond using $HgCl_2$ (11) (Figure 3B). SNOAC was not detected in the LV

of NAC-treated animals ($n = 7$). Moreover, ex vivo human blood deoxygenation in the presence of NAC in a tonometer (in nitro-gen with 5% CO_2; pH maintained at 7.3; ref. 11) resulted in both loss of SNO_{rbc} content and a nearly stoichiometric formation of SNOAC ($n = 3$; $P < 0.05$ by ANOVA followed by pairwise compari-son with the maximal value; Figure 3, C and D). The concentration of SNOAC increased as the fraction of oxygenated Hb decreased in intact erythrocytes ex vivo (Figure 3D). There was no transfer from erythrocytes to NAC when blood was maintained at 100% saturation for 15 minutes (total SNO_{rbc} was $2.1 \times 10^{-4} \pm 0.7 \times 10^{-4}$ initially and $2.0 \times 10^{-4} \pm 0.6 \times 10^{-4}$ at 15 minutes; SNOAC
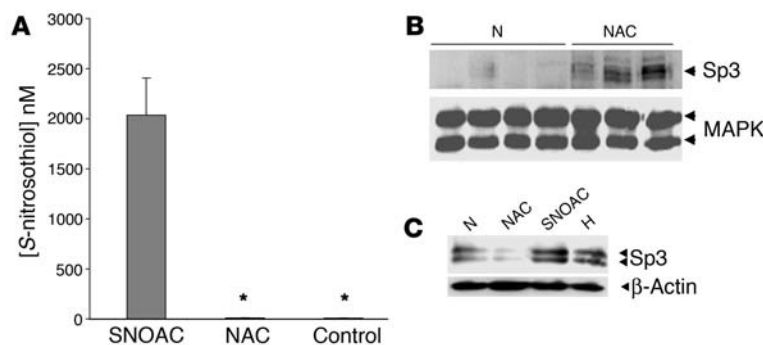
Figure 4
SNOAC recapitulates in primary pulmonary arterial endothelial cells the hypoxia-mimetic whole-lung effect of chronic NAC administration on Sp3 expression in vivo. (**A**) One micromolar SNOAC, but not 50 μM NAC, treatment (4 hours each) increased intracellular S-nitrosothiol levels (assayed by Cu/cysteine chemiluminescence; ref. 11) in primary murine pulmonary endothelial cells (*P < 0.05 compared with SNOAC treatment). (**B**) Immunoblot showing increased Sp3 expression relative to MAPK in the whole-lung homogenates of mice treated for 3 weeks with 10 mg/ml NAC but not in those of control mice. By densitometry, this increase was significant (P < 0.01). (**C**) Paradoxically, however, NAC (50 μM; 4 hours) did not increase Sp3 expression relative to β-actin in primary murine pulmonary endothelial cells in vitro, while both SNOAC (1 μM; 4 hours) and hypoxia (10%; 4 hours) did.

assayed by MS was undetectable). These findings are consistent with evidence that SNO_{rbc} distribution is, in part, dependent on oxyhemoglobin saturation (3, 11, 12) and that thiols accelerate the desaturation-induced loss of SNO_{rbc} content (11). SNOAC formation was pseudo-first order (in excess NAC) and relatively slow (k ~5.3 × 10^{-10} M/s).

We also considered that NAC could be converted to circulating SNOAC by reacting with NO in endothelial cells. S-Nitrosothiol concentrations in whole-cell lysates (43 ± 7.3 nM) were not increased by 4 hours' exposure to 50 μM NAC in the presence of 5 μM exogenous NO (45 ± 3.1 nM; P = NS; n = 3). However, cellular levels were increased by exposure to 5 μM SNOAC (2.0 ± 0.4 μM; n = 3; P < 0.05 when compared with NAC alone and control; Figure 4A).

eNOS-deficient mice are protected from the hypoxia-mimetic effects of NAC. eNOS is proposed to be important for maintaining SNO_{rbc} content (34). We studied NAC-induced PAH in eNOS$^{-/-}$ mice. The SNO_{rbc} content of these mice was 0.39 × 10^{-4} ± 0.15 × 10^{-4} (nearly a log order lower than in wild-type mice; P = 0.004). At baseline, eNOS$^{-/-}$ mice had slight increases in RV pressure and RV weight relative to those of the wild-type background (C57BL/6) mice, as reported previously (refs. 27–29; Figure 1, A and B). Strikingly, eNOS-deficient mice were completely protected from NAC-induced PAH — suggesting that the chronic effects of NAC to increase PAH are eNOS dependent — but they were not protected from SNOAC-induced PAH (Figure 1, A and B).

NAC could cause PAH in eNOS-replete mice by depleting endothelial NO or depleting eNOS-derived SNO_{rbc} (ref. 34; Figure 3). However, (a) NAC was not converted to SNOAC in eNOS-replete pulmonary endothelial cells in culture, even in the presence of exogenous NO; (b) SNO_{rbc} levels in eNOS$^{-/-}$ mice at baseline were lower than those in wild-type, NAC-treated mice; and (c) SNOAC caused, rather than ameliorating, PAH in eNOS$^{-/-}$ mice. Therefore, SNOAC excess, rather than endothelial NO or SNO_{rbc} depletion, appears most likely to have caused PAH in our model.

Chronic GSNO exposure in normoxia does not cause PAH. GSNO is an endogenous S-nitrosothiol that is similar to SNOAC in the kinetics of its homolytic decomposition (35). Normoxic C57BL/6/129SvEv mice receiving 1 mM GSNO in their drinking water for 3 weeks did not develop PAH as measured by any parameter (n = 3 in the GSNO group and 20 in the untreated control group; P = NS).

The molecular effects of S-nitrosothiols in vitro are hypoxia mimetic and recapitulate whole-lung effects of NAC in vivo. S-Nitrosothiols can have hypoxia-mimetic gene-regulatory effects involving the expression of transcription factors relevant to pulmonary vascular disease, including the hypoxia-inducible factor (HIF) (5, 13) and specificity protein (Sp) families (14). HIF 1, HIF 2, Sp1, and Sp3 are involved in regulating the expression of the genes upregulated in the lungs of mice treated chronically with NAC (9, 16, 17, 24, 25, 36). Because the expression and activity of the HIF and Sp families are affected by S-nitrosothiols in vitro (5, 13, 14, 37, 38), we tested whether chronic NAC or SNOAC could affect their pulmonary expression in mice. Interestingly, NAC increased whole-lung Sp3 expression in vivo but not in primary pulmonary vascular endothelial cells in vitro (Figure 4, B and C). SNOAC, on the other hand, increased both intracellular S-nitrosothiol levels and nuclear Sp3 expression in primary murine pulmonary vascular endothelial cells (Figure 4, A and C). Thus, conversion of NAC to SNOAC (as shown in Figure 3) could be one explanation for the paradox that NAC can increase Sp3 expression in vivo but not in vitro.

Similarly, NAC increased HIF 1 activity in whole-lung extracts in vivo (Figure 5A), though NAC suppresses upregulation of HIF 1α, the oxygen-labile subunit of HIF 1, by both substance P (31) and by oxidized low-density lipoprotein (32) in vitro. As with Sp3, conversion of NAC to SNOAC may help to explain this paradox: SNOAC and hypoxia, but not NAC, increased HIF 1α expression, relative to β actin, in primary pulmonary vascular endothelial cells in vitro (Figure 5B). This is consistent with previous work showing HIF 1α stabilization by GSNO (5, 13); indeed, we found that GSNO reduced HIF 1α monoubiquitination in bovine pulmonary arterial cells (BPAECs) expressing HA-tagged HIF 1α and a histidine-tagged, dominant-negative ubiquitin that stops ubiquitin chain propagation (K48R DN-Ub) (Figure 5C).

Mechanisms by which S-nitrosothiols inhibit HIF 1α ubiquitination and degradation are complex, involving both NO-radical reactions and transnitrosation reactions (1, 5, 13, 37–40). Low-micromolar SNOAC levels derived from NAC in vivo (Figure 3) would not generate concentrations of intravascular NO radical relevant to endothelial gene regulation (38) in the presence of millimolar concentrations of intravascular Hb (41); therefore, signaling through transnitrosation chemistry would seem more likely to be relevant to our in vivo model. NO transfer between thiols could stabilize HIF 1α through effects on prolyl hydroxylase, Akt signaling, and HIF 1α itself (13, 37–43). In addition, GSNO modifies E3 ubiquitin ligases through transnitrosation (44). Both SNOAC (Figure 5D) and GSNO (data not shown) inhibit the coimmunoprecipitation of HIF 1α with its E3 ligase, von Hippel–Lindau protein (pVHL) (45) in COS cells overexpressing both FLAG-tagged pVHL and HA-tagged HIF 1α. Strikingly, SNOAC S-nitrosylated native pVHL in HeLa cells (Figure 5E), and SNOAC and GSNO increased pVHL
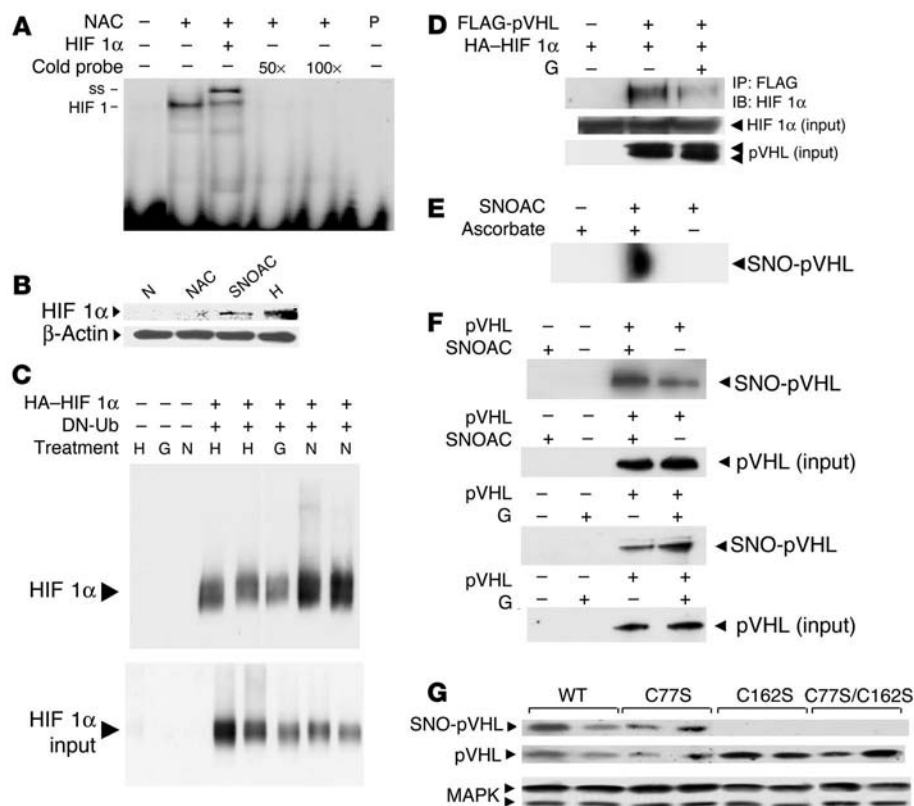
Figure 5
S-Nitrosothiols prevent normoxic ubiquitination and degradation of HIF 1α. (**A**) NAC treatment (10 mg/ml; 3 weeks) increased whole-lung HIF 1–DNA binding activity. Complexes were supershifted (ss) with anti–HIF 1β and eliminated with excess cold probe (P). (**B**) SNOAC (1 μM), like GSNO (5, 13, 38), increased normoxic HIF 1α expression in nuclear extracts isolated from primary murine pulmonary endothelial cells. NAC alone (50 μM) did not affect HIF 1α expression. β-Actin was used as a protein load control. (**C**) In BPAECs transfected with HA-tagged HIF 1α and dominant-negative His-6-Myc–tagged ubiquitin (DN-Ub), ubiquitinated proteins were isolated using a nickel column and immunoblotted for HIF 1α. Both hypoxia and GSNO (G; 10 μM) inhibited HIF 1α ubiquitination relative to normoxia. (**D**) In COS cells cotransfected with HA-tagged HIF 1α and FLAG-tagged pVHL, GSNO (10 μM) prevented the coimmunoprecipitation of HIF 1α with pVHL. (**E**) *S*-nitrosylation of pVHL by SNOAC (5 μM) in equal protein aliquots isolated from HeLa cells was identified by biotin substitution (49); in the absence of ascorbate, *S*-nitrosylated pVHL was not detected. (**F**) Similarly, SNOAC and GSNO (5 μM) increased pVHL *S*-nitrosylation in pVHL-overexpressing 786-O cells. (**G**) C162, but not C77, was identified by biotin substitution to be *S*-nitrosylated in BPAECs transfected with wild-type cysteine 77 to serine mutant (C77S), C162S, or combined C77S/C162S pVHL exposed to SNOAC (1 μM). Native pVHL and MAPK immunoblots represented the pVHL expression and protein load controls, respectively. All in vitro treatments were for 4 hours.

S-nitrosylation in 786-O cells stably overexpressing pVHL (45), in which baseline NOS activity and/or NO transfer reactions (1) also resulted in baseline pVHL *S*-nitrosylation (Figure 5F). Therefore, we studied the 2 potential pVHL *S*-nitrosylation targets (C77 and C162; refs. 45, 46). In BPAECs transiently transfected with wild-type pVHL or with cysteine-to-serine mutants C77S, C162S, or C77S/C162S, mutation of C162 eliminated SNOAC-induced pVHL *S*-nitrosylation (Figure 5G), consistent with evidence that C162 is required for pVHL to bind elongin C and ubiquitinate HIF 1α (46). Note that this effect could also reflect *S*-nitrosylation of a protein interacting with pVHL C162. Taken together, these data suggest that one element of the mechanism by which *S*-nitrosothiols may be hypoxia-mimetic involves prevention of HIF 1α ubiquitination, possibly through *S*-nitrosylation of pVHL C162. Additional stud-

ies are needed to determine the extent to which each mechanism is involved in the hypoxia-mimetic vascular effects of SNOAC in vivo.

Discussion
In vitro evidence has suggested that NO transfer reactions occurring during erythrocyte deoxygenation may signal hypoxia (3–5, 11, 12). However, the relevance of these reactions to hypoxia-associated processes at the whole-organism level has not previously been directly demonstrated. Here, we used the pharmaceutical antioxidant NAC as a bait reactant to study this biochemistry in vivo. We report that chronic NAC administration to normoxic mice can cause increased RV pressure, RV hypertrophy, and pulmonary vascular remodeling that are indistinguishable from the effects of chronic hypoxia.

NAC must be converted to SNOAC to cause PAH. Several observations suggest that the chronic pulmonary vascular effects of NAC require conversion of NAC to SNOAC. To begin with, chronic treatment with both NAC and SNOAC caused PAH (Figure 1). Thus, PAH must have resulted from 1 of 4 pathways: (a) NAC was converted to SNOAC to cause PAH; (b) SNOAC was converted to NAC to cause PAH; (c) both NAC and SNOAC caused PAH, each by a separate mechanism; or (d) the generation of a third metabolite from both NAC and SNOAC caused PAH.

Second, eNOS⁻/⁻ mice had decreased circulating SNOrbc content and were protected from NAC-induced PAH but were not protected from SNOAC-induced PAH. These data suggest that SNOAC formation from high concentrations of NAC and eNOS-derived NO, rather than reduction of lower concentrations of SNOAC to NAC, was necessary for the development of PAH. They suggest that neither NAC alone nor a common NAC/SNOAC metabolite causes PAH.

Third, we identified an oxyhemoglobin saturation–dependent mechanism by which NAC was converted to SNOAC under physiological conditions in blood. SNOAC concentrations, measured by MS, increased with the loss of SNOrbc content during whole-blood deoxygenation in the presence of NAC ex vivo (Figure 3), consistent with previously reported NO transfer from deoxygenated erythrocytes to GSH to form GSNO (3). Neither SNOAC nor GSNO (3) were formed in the presence of oxygenated blood: deoxygenation was necessary. Alternative mechanisms by which NAC could be converted to SNOAC involve reactions in which NO radical either: (a) is oxidized in nonerythrocytic cells to an NO⁺ equivalent — a slow reaction under physiological conditions — that, in turn, reacts

with NAC thiolate; or (b) reacts directly with NAC as a thiyl radical. These mechanisms are not kinetically favored in the context of competing reactions with excess heme or in the setting of hypoxia (1, 11, 40), and they were not observed experimentally in the current study: NAC had minimal reactivity with endogenous or exogenous endothelial NO. Taken together, these data suggest that erythrocyte deoxygenation resulted in conversion of NAC to SNOAC.

Fourth, NAC given systemically to the mice was converted to SNOAC in vivo, as identified by MS in RV (deoxygenated) blood. Levels of SNOAC were undetectable in LV (oxygenated) blood, suggesting that SNOAC was lost across the pulmonary vasculature and/or (consistent with our data) not formed from NAC in the presence of oxygenated erythrocytes. Further, chronic NAC administration depleted SNO_{rbc} content in vivo (Figure 3). Thus, the transnitrosation of an NO^+ equivalent from erythrocyte to NAC can explain SNOAC formation from NAC in vivo, consistent with previous observations (3, 11).

Note that these data, coupled with evidence that low levels of NO radical react minimally with thiols in isolated endothelial cells and in oxygenated erythrocytes, argue against a model in which NAC causes PAH simply by depleting endothelial NO. They also argue against a model in which SNO_{rbc} depletion causes NAC-associated PAH. Humans with hypoxia-associated PAH are deficient in S-nitrosothiol–modified Hb (34); the relevance of this deficiency to PAH in hypoxic humans is not contradicted by our data. Indeed, we show that eNOS$^{-/-}$ mice have a modest baseline increase in PA pressure associated with SNO_{rbc} depletion. However, chronic SNOAC treatment itself caused PAH in excess of that caused by SNO_{rbc} depletion.

Last, NAC-derived SNOAC is hypoxia-mimetic at the cellular level, whereas NAC itself opposes cellular effects associated with hypoxia (31, 32). SNOAC increases S-nitrosothiol levels in primary pulmonary vascular endothelial cells (Figure 4). S-nitrosothiols can upregulate hypoxia-associated genes in vitro through HIFs and/or Sp's (5, 13, 14, 38). In whole-lung homogenates, significant increases in mRNA and protein expression for genes associated with hypoxic pulmonary vascular remodeling were not uniform. This may reflect the use of whole-lung homogenates and the expression of genes in nonvascular cells. Nevertheless, mRNA and protein expression increased for several genes regulated by HIF 1, Sp3, and other hypoxia-associated factors. In the NAC-treated animals, it is unlikely that these effects on gene expression represented an effect of NAC alone: NAC (a) prevents hypoxia-mimetic cell injury in the absence of erythrocytes in vitro (31, 32); (b) did not cause PAH in the eNOS$^{-/-}$ mice (Figure 1); and (c) did not increase Sp3 or HIF 1α expression in vitro (Figures 4 and 5). Taken together, these data suggest that conversion of NAC to SNOAC was necessary for the hypoxia-mimetic effects of chronic, systemic NAC exposure.

Mechanisms by which S-nitrosothiols could be hypoxia mimetic at the cellular level. SNOAC could interact with cellular proteins to cause pulmonary vascular remodeling through: (a) homolytic cleavage to form NO radical, which reacts downstream with cellular free radicals or heme groups according to diffusion-limited kinetics; or (b) transnitrosation reactions, in which NO is transferred to a target cysteine thiol, conventionally represented as a covalent NO^+ transfer between thiolate anions (1). The interactions of S-nitrosothiols with gene-regulatory proteins such as HIF 1α are complex and likely involve both NO radical chemistry and transnitrosation chemistry (38). In the context of hypoxia and/or oxidative stress in

vitro, both NAC alone and S-nitrosothiol–derived NO radical can affect HIF 1 activation through mechanisms involving consumption of free radicals (38, 40). However, transnitrosation chemistry may be more relevant to our in vivo model because plasma S-nitrosothiols likely evolve little or no bioavailable NO radical in the presence of blood (1, 11, 41).

There are several potential targets of transnitrosation chemistry by which S-nitrosothiols might affect hypoxia-associated gene regulation. HIF 1α and prolyl hydroxylases could be modified by transnitrosation chemistry (37–39); and there may be targets in the Akt signaling cascade (13). Our results demonstrate that pVHL is also a target for S-nitrosylation. This posttranslational modification of pVHL appears to alter pVHL–HIF 1α interaction and decrease HIF 1α ubiquitination. Of pVHL's 2 critical reactive cysteines, our data suggest that C162, which is necessary for the interaction of pVHL with elongin C within the HIF-E3 ligase complex (46, 47), is the likely target for S-nitrosylation. Additional work will be required to determine which mechanisms are the most important determinants of the hypoxia-mimetic cellular effects of SNOAC in our in vivo model.

Specific pulmonary effect of SNOAC. Oral administration of GSNO, an endogenous S-nitrosothiol (35) formed during erythrocyte deoxygenation (3), did not cause PAH, though GSNO has hypoxia-mimetic effects in vitro (5, 13, 14). This suggests that GSNO toxicity is prevented by mechanisms that may be bypassed by SNOAC. GGT cleaves GSNO to S-nitrosocysteinyl glycine and glutamate, serving as a gatekeeper in certain cells to regulate intracellular GSNO effects (3). SNOAC can bypass this regulation. However, pulmonary vascular endothelial cells express functional GGT (5). Therefore, we propose that the pulmonary vasculature is protected from oral GSNO, in part, by gastrointestinal GSNO metabolism (6). Additionally, NAC and SNOAC did not affect the kidney or liver; systemic vascular beds may be insensitive to SNOAC.

Alternatively, slow transnitrosation kinetics (Figure 3) may cause SNOAC to be formed only gradually as hypoxic systemic blood returns to the right heart, favoring an effect of SNOAC on the pulmonary vascular bed, where it is consumed before returning to the systemic circulation. However, mice fed SNOAC for 3 weeks at a dose that caused PAH had no change in portal histology. Therefore, we speculate that the pulmonary vascular bed is selectively sensitive to SNOAC, perhaps reflecting differential pulmonary expression of S-nitrosothiol metabolic enzymes (6). In this regard, the pulmonary vascular bed is susceptible to remodeling in the face of increased flow, chronic inflammation, and chronic hypoxia (7); each of these conditions could lead to increased S-nitrosothiol delivery to the lung. A fuller understanding of regional S-nitrosothiol metabolism, perhaps in animals in which long-term intra-arterial S-nitrosothiols can be delivered, will likely prove to be important.

The role of eNOS in this model of PAH. eNOS has been reported to have opposing roles in PAH (18–21, 26–29). An increase in pulmonary eNOS expression, described in certain species early in the course of PAH, may have a compensatory pulmonary vasodilator role. Consistent with this paradigm, we observed a transient increase in *eNOS* mRNA as well as a later increase in eNOS protein in PAH. On the other hand, the upregulation of eNOS protein could also represent a feed-forward mechanism by which SNOAC worsens pulmonary vascular remodeling, since eNOS$^{-/-}$ mice were protected from NAC-induced PAH.

The role of eNOS in chronic PAH certainly involves effects in addition to an eNOS-associated increase in S-nitrosothiol deliv-

Table 1
Primers used for PCR experiments

Gene	Primers
HIMF (51)	Forward: 5′-GGTCCCAGTGCATATGGATGAGACCATAGA-3′
	Reverse: 5′-CACCTCTTCACTCGAGGGACAGTTGGCAGC-3′
VEGF-A (52)	Forward: 5′-CTCTACCTCCACCATGCCAAG-3′
	Reverse: 5′-GGTACTCCTGGAGGATGTCCACC-3′
Glut-1 (53)	Forward: 5′-GGTGTGCAGCAGCCTGTGTA-3′
	Reverse: 5′-AAATGAGGTGCAGGGTCCGT-3′
eNOS	Forward: 5′-AAGACAAGGCAGCGGTGGAA-3′
	Reverse: 5′-GCAGGGGGACAGGAAATAGTT-3′

ery to the pulmonary vascular bed: eNOS$^{-/-}$ mice have increased PA pressure at baseline, suggesting a role of eNOS on pulmonary vascular smooth muscle tone. These potential opposing roles for eNOS in regulating pulmonary vascular gene expression and smooth muscle tone might help to explain paradoxical observations regarding pulmonary vascular eNOS expression and activity in the setting of PAH.

Conclusions. Chronic exposure to NAC and to SNOAC causes murine PAH. We propose that these observations may be understood according to the following pathway. In normoxic, NAC-treated animals, oxyhemoglobin desaturation in erythrocytes leads to SNOAC formation in systemic blood returning to the lungs. These reactions require the presence of endogenous SNO$_{rbc}$ formed, in part, by eNOS. Formation of SNOAC — like that of GSNO, *S*-nitrosothiol-AE1, and other *S*-nitrosothiols during erythrocyte deoxygenation — is dependent on transnitrosation reactions. In these reactions, an NO$^+$ equivalent, protected from heme autocapture, is transferred from one thiolate moiety to another as Hb changes conformation during deoxygenation (1, 3, 4, 11, 12). The demonstration that SNOAC is formed during blood deoxygenation in vitro is complemented by the direct demonstration that it is present in hypoxic, but not normoxic, blood in vivo. While the cellular effects of GSNO and other *S*-nitrosothiols are regulated (1, 3, 5, 6, 14), delivery of NO to the pulmonary endothelium by SNOAC appears to bypass this regulation, causing hypoxia-mimetic effects. These observations may help to explain hypoxia-mimetic effects of systemic NAC therapy observed in humans in vivo (48).

The effect of chronic NAC exposure to cause pulmonary vascular pathology may be species dependent. It is reassuring that the NAC dose that caused PAH in mice was higher than the hypoxia-mimetic dose used in humans (48). However, PAH can be subclinical, even presenting as a terminal event (7, 8). In this context, it is noteworthy that NAC treatment may fail to decrease mortality in human clinical trials, despite beneficial antiinflammatory and antioxidant effects (30): PAH has not previously been considered as a NAC toxicity. Surveillance for this toxicity may be appropriate for long-term human trials with NAC.

In summary, these data: (a) provide the first in vivo measurements to our knowledge suggesting that NO can signal erythrocytic oxygen desaturation through *S*-nitrosothiol formation; (b) introduce a novel murine model of PAH, one in which transgenic animals are informative; (c) provide one explanation for previously reported, hypoxia-mimetic effects of NAC; and (d) suggest that PAH may be an unappreciated risk of systemic therapy with the pharmaceutical antiinflammatory and antioxidant agent NAC.

Methods

Animal protocols. C57BL/6/129SvEv, C57BL/6, and eNOS$^{-/-}$ mice (10–12 weeks old) were treated in normoxia (21% O$_2$) or hypoxia (10% O$_2$, normobaric) as described (27) with or without 10 mg/ml NAC in the drinking water for 3 weeks (achieving serum NAC levels of 16.2 ± 4.3 μM as measured by liquid chromatography/MS). The plasma NAC level at this dose was similar to the target peak level in the human study of the hypoxia-mimetic effects of NAC (48), though the dose per weight was approximately 40-fold higher and the exposure continuous. Additional mice were treated for 3 weeks with 1 mM SNOAC in the drinking water (achieving venous plasma SNOAC levels of 350 ± 34 nM). The eNOS$^{-/-}$ mice used in these studies were on a C57BL/6 background, maintained by our group and backcrossed for 10 generations (27). Protocols were approved by the University of Virginia Animal Care and Use Committee.

RV pressure measurements. A Millar Mikro Tip catheter/transducer (1.4 F) was inserted through the external jugular vein of sedated (5 mg/ml pentobarbital) mice and threaded into the RV. RV pressure was determined from the average of 10–12 measurements (PulmoDyne; Hugo Sachs Elektronik) at a stable baseline (Figure 1C).

Immunohistochemistry, vessel morphometry, and ventricular weight ratio measurements. Inflated lungs fixed in 10% formaldehyde were assessed for endothelium and smooth muscle using antibodies for von Willebrand factor and α-SMA (A0082 and M0851; Dako). Vessel morphometry was performed (>50 vessels per animal) by an investigator blinded to the treatment group. Muscularization of small (<80-μm) vessels was classified as nonmuscular, partly muscular, or muscular based on α-SMA staining. Immunostaining for 3-nitrotyrosine was performed using anti-nitrotyrosine antibody (Upstate; Supplemental Data). Cardiac tissue was dissected free from the great vessels immediately post mortem and washed with PBS. RV tissue was dissected from the septum and LV; RV weight was expressed as the ratio RV/LV+S.

NO transfer from intact erythrocytes during deoxygenation. Oxygenated, heparinized blood in a septated glass tonometer was treated with NAC (100 μM) and deoxygenated with 95% argon/5% CO$_2$. Serial samples underwent (a) co-oximetry; (b) plasma separation for SNOAC measurement by MS (see below); and (c) SNO$_{rbc}$ assay by chemiluminescence following reduction in a CuCl-saturated solution of 1 mM cysteine purged with blended argon and CO, as previously described (11).

Primary cell cultures and other cell lines. Murine lungs were harvested in PBS containing heparin (10 U/ml) and 1% penicillin/streptomycin (Invitrogen; Millipore), minced, digested in 0.3% collagenase (PBS; 37°C; 30–95 minutes), and resuspended in DMEM with D-valine (Chemicon International; Millipore) containing 20% FCS, 10 μM HEPES, 2 mM glutamine, 2 mM sodium pyruvate, 1% nonessential amino acids, 90 μg/ml heparin, 100 μg/ml endothelial growth cell factor, 1% ITS reagent, 1% penicillin/streptomycin, 50 μM 3-isobutyl-1-methylxanthine, 50 μM *N*-6-O-dibutyryl cAMP and 1 μg/ml hydrocortisone acetate. After 3 days' incubation (37°C), cells were sorted for Dil-Ac-LDL uptake (Biomedical Technologies Inc.). Positive cells were cultured for 9–12 passages. BPAECs were isolated as previously described (5). COS cells were grown to confluence in DMEM/high glucose with 10% FCS. 786-O cells (ATCC) and 780-O pVHL (gift from W. Kaelin, Dana Farber Cancer Institute, Harvard University, Boston, Massachusetts, USA) were grown in RPMI.

Immunoblotting. Immunoblot analysis on proteins isolated from lung homogenates, whole-cell extracts, and nuclear extracts was performed using antibodies against HIF1α (Novus Biologicals), Sp3, neuronal NOS, VEGF-A, MAPK (Santa Cruz Biotechnology Inc.), HIMF (gift from Dechun Li, Saint Louis University, St. Louis, Missouri, USA), fibronectin (Abcam Inc.), eNOS, iNOS (BD Biosciences), and endothelin-1 (Research Diagnostics Inc.) as described previously (5, 14).

Biotin substitution of S-nitrosothiol bonds. This was performed according to the method of Jaffrey et al. (49).

EMSA. EMSAs were performed on nuclear extracts using oligonucleotides and antibodies (for supershift) as described previously (5).

Real-time PCR. Messenger RNA was isolated from whole-lung homogenates by an RNA Easy kit (QIAGEN) as per the manufacturer's instructions. Expression of *eNOS*, *VEGF*, *HIMF*, *fibronectin*, and *Glut-1* mRNA was assessed by real-time PCR using the primers (Integrated DNA Technologies Inc.) noted in Table 1. The RT reaction was performed using 200 ng RNA under the following conditions: 25°C for 10 minutes, 48°C 30 for minutes, and 95°C for 5 minutes using the Reverse Transcriptase Kit (Applied Biosystems), as described by the manufacturer, in the presence of SYBR Green Supermix (Bio-Rad). PCR cycles for HIMF were: 95°C for 3 minutes, 95°C for 30 seconds, 63°C, 72°C for 30 seconds; for VEGF: 95°C for 3 minutes, 95°C for 30 seconds, 60°C for 1 minute, 72°C for 1 minute; for eNOS: 95°C for 3 minutes, 95°C for 30 seconds, 58°C for 1 minute, 72°C for 1 minute; for fibronectin: 95°C for 3 minutes, 95°C for 30 seconds, 55.6°C for 1 minute, 72°C for 1 minute; for Glut-1: the same conditions as for VEGF, each for 50 cycles. Relative gene expression was determined according to Livak and Schmittgen (50).

Coimmunoprecipitation. COS cells were transiently transfected with HA–HIF-1α and FLAG-pVHL using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. Following *S*-nitrosothiol treatment, cells were lysed in lysis buffer (20 mM Tris pH 7.6, 150 mM NaCl, 10% glycerol, 2 mM EDTA 1% Triton X-100, 1 mM Na_2VO_4, 1 μg/ml leupeptin, 1 mM PMSF). Lysates were incubated with Flag M2 affinity gel (Sigma-Aldrich) (2 hours; 4°C). The gel was washed 4 times in lysis buffer. Samples were eluted with 50 μl 2× SDS buffer, heated (100°C; 3 minutes), and separated by 8% SDS-PAGE. HIF 1α was identified by immunoblot (5).

Nickel column isolation. BPAECs were transfected with HA–HIF-1α and pCW-8, a His-6-Myc–tagged ubiquitin containing the K48R mutation (gift from R. Kopito, Stanford University, Palo Alto, California, USA), using Lipofectamine 2000. Untransfected and transfected cells were grown in the absence or presence of *S*-nitrosothiol (21% O_2) or in 10% O_2. Cells were lysed in the absence of EDTA, and lysates were passed over a ProBond Resin nickel column (Invitrogen) to isolate monoubiquitin-containing complexes. Complexes were eluted with imidazole and separated on 8% SDS-PAGE. HIF 1α protein was detected by immunoblot.

Site-directed mutagenesis of pVHL. pVHL was mutated using the Quick-Change Site-Directed Mutagenesis Kit (Stratagene) according to the manufacturer's instructions using the following primers: pVHL C77S primer 1, 5′-CCCTCCCAGGTCATCTTCTCTAATCGCAGTCCG-3′; primer 2, 5′-CGGACTGCGATTAGAGAAGATGACCTGGGAGGG-3′; pVHL C162S primer 1, 5′-CTGAAAGAGCGATCGCTCCAGGTTGTCCGGAGC-3′; primer 2, 5′-GCTCCGGACAACCTGGAGCGATCGCTCTTTCAG-3′.

MS. Plasma (100 μl) spiked with ¹⁵N-labeled SNOAC was applied to a Phenomenex Strata XC 33 μM SPE cartridge (60 mg, 3 ml), preequilibrated (and washed following loading) with methanol/0.02% trifluoroacetic acid in water. Analytes were eluted with methanol and concentrated under reduced pressure (0°C). Partially purified samples were reconstituted in methanol, separated on a C8 column (5 mm, 2.1 × 150 mm) (Waters 2695 HPLC) with a gradient of 0.1% formic acid in water and methanol, and analyzed by electrospray ionization MS using a Finnigan LCQ system. (Finnigan Corp.) SNOAC cations were monitored at a mass to charge ratio of 192.5–193.5.

Statistics. Multiple comparisons were made using ANOVA followed by pairwise analysis. Individual comparisons were made by Student's *t* test if normally distributed; or otherwise by Mann-Whitney rank-sum test (using SigmaStat; Jandel). Relative gene expression by RT-PCR was quantitated as above (50). Except where noted, data are presented as mean ± SD. *P* < 0.05 was considered significant.

Address correspondence to: Benjamin Gaston, Pediatric Respiratory Medicine University of Virginia Health System, Box 800386, Charlottesville, Virginia 22908, USA. Phone: (434) 924-1820; Fax: (434) 924-8388; E-mail: bmg3g@virginia.edu.

1. Gaston, B., Doctor, A., Singel, D., and Stamler, J.S. 2006. S-Nitrosothiol signaling in respiratory biology. *Am. J. Respir. Crit. Care Med.* **173**:1186–1193.
2. Kim, S.F., Huri, D.A., and Snyder, S.H. 2005. Inducible nitric oxide synthase binds, S-nitrosylates and activates cyclooxygenase-2. *Science.* **310**:1966.
3. Lipton, A.J., et al. 2001. S-Nitrosothiols signal the ventilatory response to hypoxia. *Nature.* **413**:171–174.
4. Jia, L., Bonaventura, C., Bonaventura, J., and Stamler, J.S. 1996. S-Nitrosohaemoglobin: a dynamic activity of blood involved in vascular control. *Nature.* **380**:221–226.
5. Palmer, L.A., Gaston, B., and Johns, R.A. 2000. Normoxic stabilization of hypoxia-inducible factor-1 expression and activity: redox-dependent effect of nitrogen oxides. *Mol. Pharmacol.* **58**:1197–1203.
6. Liu, L., et al. 2004. Essential roles of S-nitrosothiols in vascular homeostasis and endotoxic shock. *Cell.* **116**:617–628.
7. Farber, H.W., and Loscalzo, J. 2004. Pulmonary arterial hypertension. *N. Engl. J. Med.* **351**:1655–1665.
8. Blaise, G., Langleben, D., and Hubert, B. 2003. Pulmonary arterial hypertension: pathophysiology and anesthetic approach. *Anesthesiology.* **99**:1415–1432.
9. Semenza, G.L. 2004. O_2-regulated gene expression: transcriptional control of cardiorespiratory physiology by HIF-1. *J. Appl. Physiol.* **96**:1173–1177.
10. Jiang, B.H., Semenza, G.L., Bauer, C., and Marti, H. 1996. Hypoxia-inducible factor 1 levels vary exponentially over a physiologically relevant range of O_2 tension. *Am. J. Physiol.* **271**:C1172–C1180.
11. Doctor, A., et al. 2005. Hemoglobin conformation couples erythrocyte S-nitrosothiol content to O_2 gradients. *Proc. Natl. Acad. Sci. U. S. A.* **102**:5709–5714.
12. Pawloski, J.R., Hess, D.T., and Stamler, J.S. 2001. Export by red blood cells of nitric oxide bioactivity. *Nature.* **409**:622–626.
13. Sandau, K.B., Faus, H.G., and Brune, B. 2000. Induction of hypoxia-inducible factor 1 by nitric oxide is mediated via the PI 3K pathway. *Biochem. Biophys. Res. Com.* **278**:263–267.
14. Zaman, K., Palmer, L.A., Doctor, A., Hunt, J.F., and Gaston, B. 2004. Concentration-dependent effects of endogenous S-nitrosoglutathione on gene regulation by specificity proteins Sp3 and Sp1. *Biochem. J.* **380**:67–74.
15. Ricardo, K., Shishido, S., de Oliveira, M., and Krieger, M. 2002. Characterization of the hypotensive effect of S-nitroso-N-acetylcysteine in normotensive and hypertensive conscious rats. *Nitric Oxide.* **7**:57–66.
16. Teng, X., Li, D., Champion, H.C., and Johns, R.A. 2003. FIZZ1/RELMalpha, a novel hypoxia-induced mitogenic factor in lung with vasoconstrictive and angiogenic properties. *Circ. Res.* **92**:1065–1067.
17. Vyas-Somani, A.C., et al. 1996. Temporal alterations in basement membrane components in the pulmonary vasculature of the chronically hypoxic rat: impact of hypoxia and recovery. *Am. J. Med. Sci.* **312**:54–67.
18. Le Cras, T.D., et al. 1999. Effects of chronic hypoxia and altered hemodynamics on endothelial nitric oxide synthase expression in the adult rat lung. *J. Clin. Invest.* **101**:795–801.
19. Lam, C.-F., Peterson, T.E., Croatt, A.J., Nath, K.A., and Katusic, Z.S. 2005. Functional adaptation and remodeling of pulmonary artery in flow-induced pulmonary hypertension. *Am. J. Physiol. Heart Circ. Physiol.* **289**:H2334–H2341.
20. Xue, C., and Johns, R.A. 1996. Upregulation of nitric oxide synthase correlates temporally with onset of pulmonary vascular remodeling in the hypoxic rat. *Hypertension.* **28**:743–753.
21. Tyler, R.C., et al. 1999. Variable expression of endothelial NO synthase in three forms of rat pulmonary hypertension. *Am. J. Physiol.* **276**:L297–L303.
22. Kanazawa, F., et al. 2005. Expression of endothelin-1 in the brain and lung of rats exposed to permanent hypobaric hypoxia. *Brain Res.* **1036**:145–154.
23. Grover, T.R., et al. 2003. Intrauterine hypertension decreases lung VEGF expression and VEGF inhibition causes pulmonary hypertension in the

ovine fetus. *Am. J. Physiol. Lung Cell Mol. Physiol.* **284**:L508–L517.

24. Zhao, Y.D., et al. 2006. Microvascular regeneration in established pulmonary hypertension by angiogenic gene transfer. *Am. J. Respir. Cell Mol. Biol.* **35**:182–189.

25. Li, Q., and Dai, A. 2004. Hypoxia-inducible factor-1 alpha regulates the role of vascular endothelial growth factor on pulmonary arteries of rats with hypoxia-induced pulmonary hypertension. *Chin. Med. J.* **117**:1023–1028.

26. Hoehn, T., et al. 2003. Endothelial nitric oxide synthase (NOS) is upregulated in rapid progressive pulmonary hypertension of the newborn. *Intensive Care Med.* **29**:1757–1762.

27. Shesely, E.G., et al. 1996. Elevated blood pressures in mice lacking endothelial nitric oxide synthase. *Proc. Natl. Acad. Sci. U. S. A.* **93**:13176–13181.

28. Steudel, W., et al. 1998. Sustained pulmonary hypertension and right ventricular hypertrophy after chronic hypoxia in mice with congenital deficiency of nitric oxide synthase 3. *J. Clin. Invest.* **101**:2468–2477.

29. Fagan, K.A., et al. 1999. The pulmonary circulation of homozygous or heterozygous eNOS-null mice is hyperresponsive to mild hypoxia. *J. Clin. Invest.* **103**:291–299.

30. Hunninghake, G.W. 2005. Antioxidant therapy for idiopathic pulmonary fibrosis. *N. Engl. J. Med.* **353**:2285–2287.

31. Springer, J., and Fischer, A. 2003. Substance P-induced pulmonary vascular remodelling in precision cut lung slices. *Eur. Respir. J.* **22**:596–601.

32. Shatrov, V.A., Sumbayev, V.V., Zhou, J., and Brüne, B. 2003. Oxidized low-density lipoprotein (oxLDL) triggers hypoxia-inducible factor 1α (HIF-1α) accumulation via redox-dependent mechanisms. *Blood.* **101**:4847–4849.

33. Scharfstein, J.S., et al. 1994. In vivo transfer of nitric oxide between a plasma protein-bound reservoir and low molecular weight thiols. *J. Clin. Invest.* **94**:1432–1439.

34. McMahon, T.J., et al. 2005. A nitric oxide processing defect of red blood cells created by hypoxia: deficiency of S-nitrosohemoglobin in pulmonary hypertension. *Proc. Natl. Acad. Sci. U. S. A.* **102**:14801–14806.

35. Gaston, B., et al. 1993. Endogenous nitrogen oxides and bronchodilator S-nitrosothiols in human airways. *Proc. Natl. Acad. Sci. U. S. A.* **90**:10957–10961.

36. Wada, T., Shimba, S., and Tezuka, M. 2006. Transcriptional regulation of the hypoxia inducible factor 2alpha (HIF-2alpha) gene during adipose differentiation in 3T3-L1 cells. *Biol. Pharm. Bull.* **29**:49–54.

37. Sumbayev, V.V., Budde, A., Zhou, J., and Brüne, B. 2003. HIF-1 alpha protein as a target for S-nitrosation. 2003. *FEBS Lett.* **535**:106–112.

38. Zhou, J., and Brüne, B. 2005. NO and transcriptional regulation: from signaling to death. *Toxicology.* **208**:223–233.

39. Metzen, E., Zhou, J., Jelkmann, W., Fandrey, J., and Brüne, B. 2003. Nitric oxide impairs normoxic degradation of HIF-1α by inhibition of prolyl hydroxylases. *Mol. Biol. Cell.* **14**:3470–3481.

40. Kohl, R., Zhou, J., and Brüne, B. 2006. Reactive oxygen species attenuate nitric-oxide-mediated hypoxia-inducible factor-1alpha stabilization. *Free Radic. Biol. Med.* **40**:1430–1442.

41. Jeffers, A., et al. 2005. Hemoglobin mediated nitrite activation of soluble guanylyl cyclase. *Comp. Biochem. Physiol. A Mol. Integr. Physiol.* **142**:130–135.

42. Masson, N., Willam, C., Maxwell, P.H., Pugh, C.W., and Ratcliffe, P.J. 2001. Independent function of two destruction domains in hypoxia-inducible factor-alpha chains activated by prolyl hydroxylation. *EMBO J.* **20**:5197–5206.

43. Ivan, M., et al. 2001. HIFα targeted for VHL-mediated destruction by proline hydroxylation: implications for O_2 sensing. *Science.* **292**:464–468.

44. Yao, D., et al. 2004. Nitrosative stress linked to sporadic Parkinson's disease: S-nitrosylation of Parkin regulates its E3 ligase activity. *Proc. Natl. Acad. Sci. U. S. A.* **101**:10810–10814.

45. Lonergan, K.M., et al. 1998. Regulation of hypoxia-inducible mRNAs by the von Hippel-Lindau tumor suppressor protein requires binding to complexes containing elongins B/C and Cul2. *Mol. Cell. Biol.* **18**:732–741.

46. Ohh, M., et al. 2000. Ubiquitination of hypoxia-inducible factor requires direct binding to the beta-domain of the von Hippel-Lindau protein. *Nat. Cell Biol.* **2**:423–427.

47. Stebbins, C.E., Kaelin, W.G., Jr., and Pavletich, N.P. 1999. Structure of the VHL-ElonginC-ElonginB complex: implications for VHL tumor suppressor function. *Science.* **284**:455–461.

48. Hildebrandt, W., Alexander, S., Bartsch, P., and Droge, W. 2002. Effect of N-acetyl-cysteine on the hypoxic ventilatory response and erythropoietin production: linkage between plasma thiol redox state and O(2) chemosensitivity. *Blood.* **99**:1552–1555.

49. Jaffrey, S.R., Erdjument-Bromage, H., Ferris, C.D., Tempst, P., and Snyder, S.H. 2001. Protein S-nitrosylation: a physiological signal for neuronal nitric oxide. *Nat. Cell Biol.* **3**:193–197.

50. Livak, K.J., and Schmittgen, T.D. 2001. Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta C_T}$ method. *Methods.* **25**:402–408.

51. Loke, P., Nair, M.G., Parkinson, J., Guiliano, D., and Allen, M.E. 2002. IL4-dependent alternatively-activated macrophages have distinctive in vivo gene expression phenotype. *BMC Immunol.* **3**:7.

52. Chintalgattu, V., Nair, D.M., and Katwa, L.C. 2003. Cardiac myofibroblasts: a novel source of vascular endothelial growth factor (VEGF) and its receptors Ft-1 and KDR. *J. Mol. Cell. Cardiol.* **35**:277–286.

53. Tang, Y., et al. 2006. Effect of hypoxic preconditioning on brain genomic response before and following ischemia in the adult mouse: identification of potential neuroprotective candidates for stroke. *Neurobiol. Dis.* **21**:18–28.

S-nitrosoglutathione Reductase, Gender, and the Development of Pulmonary Arterial Hypertension

Palmer LA, *Que L, Brown-Steinke K, deRonde K, Gaston B
University of Virginia Health System, Charlottesville VA 22908
*Duke University Medical Center, Durham NC

Nitric oxide bioactivity, mediated through the formation of S-nitrosothiols (SNOs), has a significant influence on vascular tone. Moreover, numerous components that regulate SNO bioavailability in the pulmonary vasculature display gender dependent differences in activity and/or expression. Pulmonary arterial hypertension (PAH) is a condition with known gender disparity. To date, little research has focused on the mechanism(s) mediating this gender discordance. Therefore, we examined the hypothesis that S-nitrosothiol bioavailability, mediated through the actions of S-nitrosoglutathione reductase (GSNO-R), may play a role in the gender predilection seen in this disease. Using a novel mouse model of PAH induced by N-acetyl cysteine (Palmer et al. J. Clin Invest. 2007), our data reveal female mice have higher resting right ventricular pressures and right ventricular weights than males, but are protected from the N-acetyl cysteine-induced increases observed in males. Castration eliminated this response. S-nitrosylated N-acetylated cysteine (SNOAC) levels present in the serum of N-acetyl cysteine-treated animals was greater in the male animals, suggesting that differential S-nitrosothiol metabolism may be responsible for these observations. Analysis of the activity and expression of GSNO-R demonstrated: 1) GSNO-R activity was greater in female compared to the male animals with no changes in mRNA or protein expression. 2) Castration elevated GSNO-R activity to levels seen in female animals. 3) GSNO-R is S-nitrosylated. Taken together, the data suggest that changes in GSNO-R activity, possibly mediated by S-nitrosylation, may, in part, contribute to the gender-specific development of PAH induced by the delivery of S-nitrosothiols to the pulmonary vasculature.

Specific Aims:
Pulmonary arterial hypertension (PAH) is a condition with known gender disparity. We propose that sex-linked discrepancies in the bioactivities of S-nitrosothiols (SNOs), which are potent regulators of pulmonary arterial pressure, play an important role in the gender divergence seen in this disease. The vascular bioactivity of SNOs is determined by three components: formation, delivery from the red blood cell, and metabolism. The formation of SNOs is affected by endothelial nitric oxide synthase (eNOS) activation; SNO efflux from the red blood cell is largely determined by oxygen saturation; and SNO metabolism is regulated, in part, by the activity of S-nitrosoglutathione reductase (GSNO-reductase). There are gender differences in all these regulatory mechanisms, which are generally greater in females than in males. Using a novel mouse model of PAH induced by unregulated delivery of SNOs to the pulmonary vasculature, our data reveal female mice had higher resting right ventricular pressures than males, but are protected from the additional SNO-induced increases observed in males. Castration eliminated this difference. GSNO-reductase appears to be fundamental to the observed gender differences since GSNO-reductase activity in castrated mice was equal to that of female mice, and overexpression of GSNO-reductase altered eNOS phosphorylation at serine 1177, a residue implicated in eNOS activation. Based on these observations, **we hypothesize 1) disruption of the eNOS-GSNO-reductase system plays an important role in one gender compared to the other with respect to the development of PAH and 2) disruption of the delicate balance between eNOS and GSNO-reductase activities, which maintain a steady state of SNO bioactivity, contribute to the gender specific differences seen in PAH.**

Specific Aim 1 tests the hypothesis that crosstalk between GSNO-reductase and eNOS contributes to S-nitrosothiol bioactivities seen in the in the pulmonary vasculature.
1a.) Measure the gender differences in the expression and activity of GSNO-reductase in mice.
1b.) Determine the influence of GSNO-reductase on the expression and activity of eNOS.
1d.) Determine the influence of eNOS on the expression and activity of GSNO-reductase.
1c.) Identify the subcellular compartment(s) where GSNO-reductase and eNOS interact

Specific Aim 2 tests the hypothesis that the gender discordance seen in pulmonary arterial hypertension is due to alterations in GSNO-reductase activity.
2a.) Determine the mechanism by which sex steroids alter the expression and/or activity of GSNO-reductase.
2b.) Determine if GSNO-reductase polymorphisms are associated with the development of pulmonary arterial hypertension in humans.
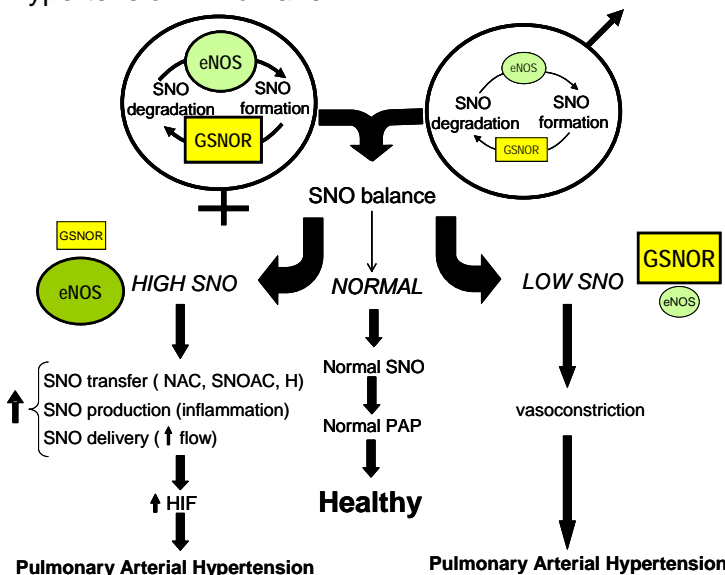

Figure 1. Mismatch between SNO-production/ delivery and SNO-metabolism may contribute to the development of PAH. The importance of maintaining a balance between SNO production and degradation is illustrated in the schematic diagram. The relative expression and/or activity of eNOS and GSNO-reductase are different in male and female mice, with females having increased expression and/or activity of both proteins. An imbalance in SNO bioavailability in the pulmonary vasculature could lead to the activation of downstream events that contribute to the development of pulmonary arterial hypertension. This proposal examines the role GSNO-reductase activity and/or expression plays in the gender discordance seen in this disease.

In summary, we hypothesize SNO bioactivity is regulated by the eNOS-GSNO-reductase system, that sex hormones modulate the activity/expression of both enzymes and that altered SNO bioavailability resulting from a disruption in the balance between eNOS and GSNO-reductase activities causes PAH. Although blood SNO levels are similar in male and female mice, eNOS and GSNO-reductase activities are increased in females. As such, we hypothesize that disturbances in the eNOS-GSNO-reuctases system (ie SNO production/turnover rate), may have a more drastic consequence in females than in males.